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HPLC METHOD FOR AMINO ACIDS PROFILE IN BIOLOGICAL FLUIDS AND INBORN METABOLIC DISORDERS OF AMINOACIDOPATHIES

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ABSTRACT

Quantification of total and individual amino acids in biological fluids such as plasma, urine and cerebrospinal fluid has an important diagnostic implication in laboratory medicine. The present paper describes protocols for the assay of total amino acids by modified method based on dinitrophenyl and HPLC profile involving pre-column derivatization with o-pthalaldehyde (OPA) derivatization, respectively. The method, based on the alkylation of -SH groups prior to OPA derivatization of amino acids followed by reverse phase high performance liquid chromatography, provide a comprehensive profile of more than twenty amino acids (including -SH group containing) in a single run lasting about 45 minutes. The present study, apart from establishing the normal profile of amino acids in plasma of Indian sub population, also presents HPLC profile for some of the rare amino acidopathies.

KEY WORDS

O- pthalaldehyde, amino acidopathies, amino acids profile, reverse phase HPLC, Inborn metabolic disorders.

INTRODUCTION

Assay of total as well as individual amino acids level in biological fluids has become an increasing practice in laboratory medicine (1-3). While the total amino acids content of plasma and urine is expected to reflect the nutritional/metabolic status, the individual amino acids profile of biological fluids are of importance in confirming or other wise to rule out the suspected amino acidopathies. Early detection of inborn metabolic disorders (IMDs) of amino acidopathies often helps in therapeutic intervention, which would prevent the irreversible damage or attenuate the severity of the clinical manifestation. Further, quantification of individual amino acids is of importance in monitoring the therapeutic intervention by way of dietary manipulations and/or vitamin supplement therapy. In view of the diagnostic significance of amino acids profile in biological fluids, a method that would provide a comprehensive profile of most of the amino acids is of immense use in clinical laboratories. In this regard, though the HPLC methods based on OPA derivatization offer an opportunity to quantify most of the amino acids they often fail to detect amino acids with –SH groups (4,5). The importance of –SH group containing amino acids, in particular plasma homocysteine and its relation to cardio/cerebro-vascular disorders (4-8), in laboratory medicine has lead to a spurt of interest in the development of methods for the assay of homocysteine in plasma (9-15). However, the methods based on immuno assays (9), enzymatic (10), electrochemical measurement (11) and –SH group specific fluorescent reagents (13-14) have limited use to detect a given amino acid alone or at the maximum –SH group containing amino acids, but not other amino acids. Present paper describes a HPLC method based on alkylation of –SH groups and OPA derivatization, which would provide a comprehensive profile of most of the amino acids (inclusive of -SH groups containing) in biological fluids such as plasma/urine and CSF. To the best of our knowledge, this happens to be the first attempt to establish amino acids profile in plasma of an Indian sub population based on HPLC method. Further, the present report also provides HPLC profile for some of the rare inborn metabolic disorders (IMDs) of amino acids confirmed at our center.

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MATERIALS

All the chemicals used were of analytical grade. Sodium borate buffer: 0.5 M , pH 10.5; Dinitrofluorobenzene (DNFB) stock solution: 325 µl of DNFB (Sigma Chemical Co, USA) was dissolved in 25 ml of acetone (stored at 4°C in a brown colored glass bottle); Working DNFB reagent: Prepared by diluting 1 volume of stock DNFB reagent with 9 volumes of 0.155 M borate buffer just before use; Ortho-pthalaldehyde (OPA) reagent: Prepared, just before use, by dissolving 10 mg of OPA (Sigma Chem. Co., USA) in 1 ml of methanol-borate buffer (1:9 v/v) mixture containing 0.01% (v/v) β-mercaptoethanol (Sigma Chem. Co, USA); Alkylating reagent: 0.2 M iodoacetate (Sigma Chem. Co, USA) in 0.5 M borate buffer, pH 10.5. Reference standards: Stock standards (5 mM) of individual amino acids (Sigma Chem. Co USA) were prepared in methanol : water mixture (8:2 v/v) and stored at 4°C. Reference standard for total amino acids (1mM each of glutamate and glycine solution): Prepared by mixing 1 ml each of 5 mM glutamate and glycine and made up to 5 ml with methanol water mixture (80:20 v/v); Cocktail of standard amino acids (for HPLC profile): Equimolar concentration (0.1 mM of individual amino acids) mixture was prepared just before use by mixing the stock solutions of individual amino acids. Individual standards for asparagine and glutamine were prepared freshly and incorporated with the standard amino acids mixture just before derivatizing with OPA reagent. Retention time for histidine was established separately by using reference standard.

Specimens collection and processing.

Adults (n=34) of 20 - 45 years of age group, without any apparent symptoms of illness, constituted the control group for this study. Patient materials comprised subjects attending NIMHANS clinics for diagnosis of IMDs with suspected amino acidopathies and referrals from other centers. Overnight fasting blood samples were collected in heparinized syringe from anti-cubital vein and immediately (within twenty minutes) centrifuged (3000 rpm /15 minutes) to separate the plasma.

Urine/CSF specimens were centrifuged (3000 rpm/ 15 minutes) to clear the cells /debris, if any. An aliquot of the supernatant from urine was subjected for creatinine assay in an auto analyzer (Hitachi 911) as per the analysis protocol of the manufacturer of the reagents (Boehringer and Mannheim).

Reduction of disulphide compounds and deproteinization of biological fluids:

Plasma/urine/CSF specimens (0.1 ml) were mixed with (5 µl) β-mercaptoethanol (β-ME) and allowed to stand for 5 minutes at room temperature followed by precipitation with ice-cold methanol (395 µl) while vortexing. Tubes were allowed to stand for 15 minutes in an ice bucket before centrifuging (5000 rpm / 15 minutes) and the supernatant was collected. Efficiency of protein precipitation step was assessed by Bradford's dye binding method (16). The protein free supernatants were processed immediately for assaying total amino acids and HPLC analysis or stored at -70° C until further analysis.

Assay of total amino acids:

Total amino acids content of protein free supernatants were estimated by modified dinitrophenyl (DNP) derivatization method (17). In brief, the methanol extract (100 µl) of biological fluid or the reference standard (equimolar mixture of glutamate:glycine) was made up to 250 µl with 80% (v/v) methanol. Equal volume (250 µl) of borate buffer was added to each of the tubes, followed by 0.5 ml of DNFB reagent. Tubes were incubated at 45°C for 30 minutes and allowed to attain room temperature. After adding 1 ml of 0.25 M HCl to each of the tubes and mixing, the absorbancy was measured at 420 nm. Apart from reagent blank and reference standards, some of the samples also carried a known amount of glutamate:glycine mixture to assess the recovery of total amino acids through different steps of assay procedure.

Alkylation of –SH compounds and pre-column derivatization with O-pthalaldehyde and reverse phase separation:

An equimolar concentration (0.1 mM) of individual or cocktail of amino acids mixture or plasma/urine or CSF extract (≡2000 ±100 picomoles of total amino acids) of methanol (0.1 ml) were mixed with (50 µl) borate buffer (0.5 M, pH 10.5) containing iodoacetate (0.2 M) followed by OPA reagent (25 µl). Derivatized solution was made up to 1 ml by adding 825 µl start eluent (mixture of acetate buffer, 0.05 M, pH 6.8:methanol 4:2 v/v). OPA derivatized amino acids were injected to HPLC system (Shimadzu, Japan) equipped with 20 µl (100 µl for CSF extract) injection loop and C18 column (25 cm) fitted with a guard

column (1 cm) housed in an incubator oven (Mayura Analytical Pvt Ltd, Bangalore) set at 40°C constant temperature. Individual amino acids were separated by reverse phase (gradient of 0.05 M acetate buffer, pH 6.8 (solvent A) and methanol (solvent B) as given in table1), with a flow rate of 1.0 ml per minute. Resolution of amino acid derivatives were monitored through fluorescence detector with excitation and emission set at 330 nm and 450 nm respectively.

Peak identification and quantification:

Amino acids were detected based on the retention time established for the individual amino acid under defined experimental conditions. Linearity of the peak areas for different concentrations, ranging from 20 - 200 picomoles, of individual amino acids were determined. Calculation was based on the area under peak established for a given amino acid of known concentration. Further, whenever a peak from test sample required to be confirmed for a given amino acid, HPLC was repeated after spiking the amino acid (s) of interest to the sample.

Experiments to establish the recovery for amino acids mixture through different phases including precipitation step was carried out through the procedure and the percent of recovery was assessed.

RESULTS

Protein content of the methanol extract was found to be less than 6 μg/ml implying the efficacy of deproteinization brought about by methanol precipitation. Recovery of amino acids, incorporated at the level of precipitation step, was found to be 97-101 percent as determined by DNP derivatization method for total amino acids. The OPA derivatives of amino acids were found to have differential sensitivity, in terms of relative fluorescence for an equimolar concentration of individual amino acids, with low and high sensitivity to histidine (not shown) and taurine respectively (Fig1). The observed differential sensitivity to OPA derivatives of different amino acids made us to establish standard curve for individual amino acid(s) to ascertain the linearity over a range of concentrations (Fig 1).

OPA derivatization of -SH group containing amino acids, in the absence of alkylation were found to be non-fluorescent, such as cysteine and homocysteine could not be detected. However, prior alkylation of -SH groups with iodoacetate, as suggested by Turnell et al (18), has rendered -SH group containing amino acids fluorescent following OPA derivatization. Thus, the described HPLC method is able to resolve and quantify most of the amino acids present in biological fluids within a total run period of 45 minutes. With the solvent gradient system as given in Fig 2,(A, B) we could establish the normal range of amino acids in plasma for an Indian subpopulation (Fig 2, table 1), the profile being comparable to western population reported in literature (19). Method, by changing over to injection loop of 100 μl capacity, was also employed to estimate amino acids content of CSF (Fig 3). Further, we were able to confirm the diagnosis of varieties of amino acidopathies such as phenylketonuria, hyperornithinemia, homocysteinemia, homocystinuria, hyperargininemia, maple syrup urine disease (MSUD) and non-ketotic hyperglycinemia (Figs 4 -10). Incidentally, it was also noted that the gradient system recommended in the main method fails to resolve citrulline from glycine (Fig.11 A, B), which called for the alteration in the solvent gradient. Accordingly, a different solvent gradient system was evolved to separate these two amino acids, which has enabled us to confirm a case of citrullinemia (Fig. 11 C, D).

DISCUSSION

The assay of total and the individual amino acids in biological fluids is of importance in laboratory medicine. Accurate estimation of amino acids in biological fluids depends on several aspects of the procedure such as efficiency of extraction, the specificity and sensitivity of the method employed. The extraction step of amino acids from biological fluids, apart from precipitation of proteins, should also be effective in extracting the bound forms of amino acids. Further, the method should be able to resolve all the amino acids and also sensitive enough to quantify them. In the present context, disulphydral derivatives of cysteine and homocysteine and their mixed disulphides need to be reduced and released from bound fraction before their quantification. Treating the specimens with β–ME prior to methanol precipitation, to reduce the disulphydral compounds to respective –SH compounds, followed by the subsequent alkylation of the –SH groups with iodoacetate rendered them fluorescent upon OPA derivatization. Further the use of methanol, the efficacy of which has been validated and recommended in the present method, has eliminated additional neutralization step before DNP and OPA derivatization for the assay of total

and individual amino acids profile by spectrophotometric and HPLC method(s) respectively. The recovery (97-101%) of the amino acids through the protein precipitation step and almost protein free (< 6 μg protein/ml supernatant) nature of the methanol extract of plasma is a testimony to the efficacy of the methanol precipitation in lieu of acid reagents. It may also be noted that the described modified method for total amino acids assay, by avoiding the use of a solvent such as acid dioxane described in earlier methods based on DNP derivatization (17), is less cumbersome.

Often the methods based on pre column derivatization with OPA and reverse phase HPLC, because of their limited specificity to certain groups of amino acids; fail to provide a comprehensive profile of amino acids. In particular, the OPA derivatives of

–SH group containing amino acids are not fluorescent (18) enough to be detected. In this context it is of relevance to note that metabolic disorders of transullfuration of amino acids in general has attracted the attention of laboratory medicine (23). In particular, homocysteine is increasingly implicated in both cerebro and cardio vascular disorders (4-8). The fact that homocysteine and methionine are metabolically linked to each other, the study of either of them alone may not provide meaningful information. Further, when protein challenge and/or specific amino acid(s) (such as methionine) loading test needs to be carried out, a comprehensive amino acids profile would be of more informative than any given amino acid alone. Thus, the alkylation of –SH groups of amino acids by iodoacetate prior to derivatization with OPA as described in the present method enabled us to have a comprehensive profile of amino acids, including cysteine and homocysteine (except for proline and hydroxyproline), present in biological fluids. The observed differential sensitivity (Fig.1) of various amino acids of an equimolar concentration, in terms of relative fluorescence of OPA derivatives, makes it imperative to establish linearity range for individual amino acid(s) for calculation of area corresponding to respective peaks. The present method has established the linearity for amino acids at different concentrations i.e., 20, 50,100 (Fig 1) and 200 (not shown) picomoles per injection.

The main method described, with solvent gradient system as given in Fig. 11 A, could separate most of the amino acids within a total run period of 45 minutes. Our findings on normal range for plasma amino acids profile for adults (Table 1) corroborates with that of western population published in the literature (19). Over the period, extending from 1994-2000, we have been able to confirm many of the rare amino acidopathies (Table 2) and also to extend the laboratory back up to monitor the outcome of therapeutic intervention in case of PKU, hyperornithinemia and homocysteinemia. Further, employing solvent gradient as given in Fig. 11 (A,B), an altered protocol at the cost of resolution of other amino acids, we were able to separate glycine from citrulline and confirm the diagnosis of a case of citrullinemia (Fig.11). Presently, we are working on a common solvent gradient system to resolve all the amino acids, including citrulline, in a single run of much shorter total run time.

Though CSF amino acids profile is rarely analyzed for diagnostic purpose, in certain situations such as suspected case of non-ketotic hyperglycinemia, the ratio of CSF to serum glycine would be of confirmative in nature (20). Further, plasma and urine amino acids profile in hyperornithinemia (Fig 5 A,B) and homocystinuria (Fig. 7), apart from confirming the diagnosis of the respective metabolic disorders may as well provide clues for rational therapeutic intervention. Thus, the observed increase in urinary excretion of lysine in hyperornithinemia (Fig 5) with the concomitant decrease in serum level appears to be due to the excessive plasma ornithine competing with lysine for the reuptake from renal ultrafiltrate. The combined effect of enhanced excretion in renal tubules and the dietary restriction with respect to the protein intake, could explain the observed low level of plasma lysine, one of the nutritionally essential amino acids. This observation supports the rationale for lysine supplementation, along with vitamin B6, in hyperornithinemia. The fact that both ornithine and lysine compete for the same reuptake mechanism in renal tubules (21), the supplementation of lysine in essence should facilitate the excretion of ornithine the excessive level of which is attributed to the gyrate atrophy of choroid and retina associated with hyper ornithinemia.

In case of homocysteinemia and homocystinuria, the HPLC profile of amino acids in plasma and urine (Figs 6 and 7) is of use in differential diagnosis of the two. Homocysteinemia (Fig 6) is characterized by relatively moderate increase in plasma homocysteine (19.2 μM) where in urinary excretion

is within the normal range. In addition to this, plasma cysteine level (290 μM) is found to be within the normal range. On the other hand homocystinuria (Fig7) is characterized by an elevated level of homocysteine in both plasma (192 μM) and urine (1097 μMoles/g creatinine). Further, unlike in homocysteinemia, a significant depletion of cysteine in plasma (almost undetectable) is also noted in homocystinuria (Fig 7). The relative amount of cysteine to that of homocysteine would suggest severity of the metabolic block in the conversion of homocysteine to cystathionine and/or reconversion to methionine.

It is to be noted that in spite of better resolution, sensitivity and reproducibility of retention time for a given amino acids under defined conditions, it is not unusual to find some other compound of known or unknown co-eluting with one or the other amino acids. Hence the diagnosis of IMDs of amino acids based on HPLC profile needs to be substantiated with other laboratory findings such as plasma ammonia, acid base parameters as well as clinical history including the medications, if any. One such experience of ours relates to a case of citrullinemia which would have been reported as hypergycinemia, but for the high plasma ammonia which suggested the possibility of urea cycle disorder. As it turned out, citrulline was found to have same retention time as that of glycine under the solvent gradient applied (Fig. 11 A) and co-eluted with the latter. However, an elevated plasma ammonia made us to look out for intermediate metabolites of urea cycle, where in citrulline was found to be elevated (Fig 11). That experience made us to develop a different solvent gradient system (Fig. 11 B) to resolve citrulline and glycine from each other. Apart from confirming the case as citrullinemia, the plasma glycine content was in fact found to be depleted as a result of patient having administered with sodium benzoate prior to refer the specimen for investigation.

To summarize, the present study, by alkylating the –SH groups with iodoacetate prior to derivatization with OPA reagent made it possible to quantify cysteine and homocysteine in addition to most of the other amino acids. Our experience makes us to believe that by adding the HPLC method to the armamentarium of diagnostic facilities would lead to the diagnosis of more cases of IMDs pertaining to amino acidopathies, which in turn could be of importance to both therapeutic and preventive medicine. Availability of such facilities in neonatal/ paediatric care centers, by way of an early diagnosis and therapeutic intervention, may help in prevention of irreversible damage and/or attenuation of clinical severity of the disorder. In certain instances where therapeutic intervention is not feasible, confirmed diagnosis could as well help in genetic counseling to prevent the recurrence of childbirths with amino acidopathies and associated medical complications as well as emotional/economical burden to the afflicted family.

Table 1: Plasma amino acids profile of a normal adult Indian sub population (n= 34) compared with Western population.

Amino acids(s)	Plasma concentration (μM)			Literature*
	Range	**Mean**	**SD**	Range (μM)
Glycine	134 – 466	266.0	76.1	121 – 401
Alanine	134 – 597	339.0	113.3	203 – 518
Valine	112 – 242	168.9	31.0	168 – 350
Leucine	53 – 135	77.4	18.2	73 – 172
Isoleucine	38 – 94	60.0	14.1	42 – 98
Serine	50 – 201	116.4	38.5	67 – 161
Threonine	30 – 256	121.5	49.5	74 – 175
Aspartate	5 – 43	16.9	10.6	0 – 9
Glutamate	8 – 99	40.8	30.9	12 – 98
Asparagine	8 – 83	52.7	15.8	36 – 71
Histidine	18 – 155	68.5	34.4	48 – 121
Arginine	26 – 123	73.5	22.9	50 – 126
Lysine	54 – 240	156.5	41.8	107 – 244
Ornithine	25 – 153	65.6	31.5	24 – 112
Phenylalanine	27 – 115	63.2	21.6	40 – 81
Tyrosine	7 – 127	61.2	26.1	43 – 90
Tryptophan	32 – 85	54.7	15.7	24 – 72
Cysteine (total)	245 – 332	222.9	60.0	268 – 300
Homocysteine (total)	3.8 – 19.0	8.4	4.6	6 – 16
Taurine	21 – 212	84.2	49.8	38 – 197
Methionine	13 – 38	25.0	6.2	13 – 33

* Teerlink Tom *et al* (1994) See Ref. # 19

Table 2: Varieties of IMDs of amino acids confirmed during the period 1994 - 2000

Amino acidopathies	No
Phenylketonuria	11
Maple Syrup Urine Disease	7
Neonatal tyrosinemia	4
Ketotic hyperglycinemia	8
Non-ketotic hyperglycinemia	2
Citrullinemia	2
Hyperargininemia	2
Hyperornithinemia (associated with gyrate atrophy of the choroid and retina)	4
Hyperprolinemia, type I	2
Homocystinuria	1
Homocysteinemia	4
Sulfite oxidase defect	1
Total	**48**

LEGENDS FOR FIGURES

Figure1: HPLC profile(s) of a mixture of an equimolar concentration of various amino acids ranging from 20 – 100 picomoles (A: 20 picomoles; B: 50 picomoles; C: 100 picomoles), with solvent gradient system as indicated in table 1. Note the difference in the total area under peak for an equimolar concentration of different amino acids. Peaks 1-24 represent different amino acids of 100 picomoles each (1: Asp; 2: Glu; 3: Cys; 4:Asn; 5: Hcy; 6: Ser; 7: Gln; 8: His (not shown); 9: Gly; 10: Thr; 11: Arg; 12: Tau; 13: Ala; 14: Tyr; 15: Trp; 16: Met; 17: Val; 18: Phe; 19: Ile; 20: Leu; 21: Orn; 22: Lys.), eluted at different retention time in minutes.

Figure 2: HPLC profile of amino acids of a normal adult plasma (A) and urine (B). Panels C and D represent plasma and urine amino acids profile of a neonate (9 days old). Note a small (due to insensitivity of His-OPA derivative) peak (#8) representing His, with the retention time of 19.9 ± 0.04 minutes is also seen in both adult and neonate plasma. Also note, the high content of taurine in plasma and urine of child as compared to adult.

Figure 3: Amino acids profile of normal (with no neurological complications/symptoms) adult CSF: Peaks 1: Asp (2.0); 2: Glu (12.1); 4: Asn (11.0); 6: Ser (21.0); 7: Gln (235.0); 9: Gly (38.0); 10: Thr (7.0); 11: Arg (13.0); 12: Tau (3.0); 13: Ala (52.0); 16: Met (1.2); 17: Val (33.0); 18: Phe (8.2); 19: Ile (10.8); 20: Leu (13.5); 21: Orn (2.5); 22: Lys (17.0). Numbers in parenthesis indicate respective amino acids concentration in μMoles/L.

Figure 4: Plasma (A) and urine (B) amino acids profile of a phenylketonuria (PKU, hyperphenyalaninemia with ketonuria) case. Note an elevated peak (# 18) of phenylalanine in plasma (650 μM) and urine (2656 μMoles/g creatinine). Also note undetectable peak (# 14) for Tyr in both plasma and urine.

Figure 5: HPLC profile of amino acids in plasma (A) and urine (B) in hyper-ornithinemia. Note the elevated level of ornithine both in plasma (2290 μM) and urine (1415 μMoles/g creatinine). Also note the increase in the urinary excretion of lysine (Panel B, peak # 22, 272 μMoles/g creatinine) with a concomitant depletion of lysine in plasma (panel A, peak # 22). Note the significant decrease in both ornithine and lysine content of plasma (C) and urine (D) following protein restricted diet and vitamin B6 supplementation.

Figure 6: Amino acids profile of plasma (A) and urine (B) of a homocysteinemia (24 yrs) case. Note the relatively large peak (#5) for homocysteine in plasma (19.2 μM) of homocysteinemia case (inset D) compared to a normal (inset C). Note the normal level of plasma cysteine (287 μM) and Met (24 μM) content. Also note the almost undetectable homocysteine in urine (B).

Figure 7: Amino acids profile of plasma (A) and urine (B) of homocystinuria case. Note the elevated level of homocysteine (peak #5) in both plasma (192 μM) and urine (1097 μMoles/g creatinine). Also note the low content of cysteine (peak# 3), almost undetectable, in both plasma and urine.

Figure 8: Amino acids profile of plasma (A) and urine (B) of maple syrup urine disease (6 days old child) characterized by elevated levels of branched chain amino acids in plasma and urine and ketonuria . Note the elevated level of branched chain amino acids in both plasma (Val, 386 μM; Ile, 463 μM; and Leu, 813 μM) and urine (Val:415; Ile:463 and Leu:1559 μMoles/g creatinine)

Figure 9: Amino acids profile of plasma (panel A) and urine (panel B) of a argininemia case, due to arginase deficiency (confirmed by assaying RBC arginase activity). Note an elevated arginine (peak # 11) content in plasma (550 μM) but normal level in urine (22 μMoles/g creatinine).

Figure 10: Plasma (panel A) and urinary (panel B) amino acids profile of non-ketotic hyperglycinemia, apparently due to deficiency of glycine cleavage system. An elevated level of Gly (peak # 9) in both plasma (2579 μM) and urine (5731 μMoles/g creatinine) is discernible, which was brought down by administering sodium benzoate (results not shown).

Figure 11: Co-elution of glycine and citrulline as per the main method (panels A and C) of plasma and urine respectively, and their resolution by altering the gradient of the solvent system (Panels B and D). Panels A and C represent plasma and urinary amino acids profile(s) of a citrullinemia case as per the main method with solvent gradient as given in panel A. Note the peak # 9 representing Gly, with no extra peak for citrulline, the amino acids profile appear to be normal because of co-elution of citrulline with glycine. Separation of citrulline (Peak # 23) from glycine (peak # 9), in plasma (B) and urine (D) of the same case with different retention time, by altering solvent gradient as given in panel B. Also note the unusual low level of plasma Gly (102 μM) peak (# 9) as a result of sodium benzoate treatment prior to referring the sample for diagnosis.

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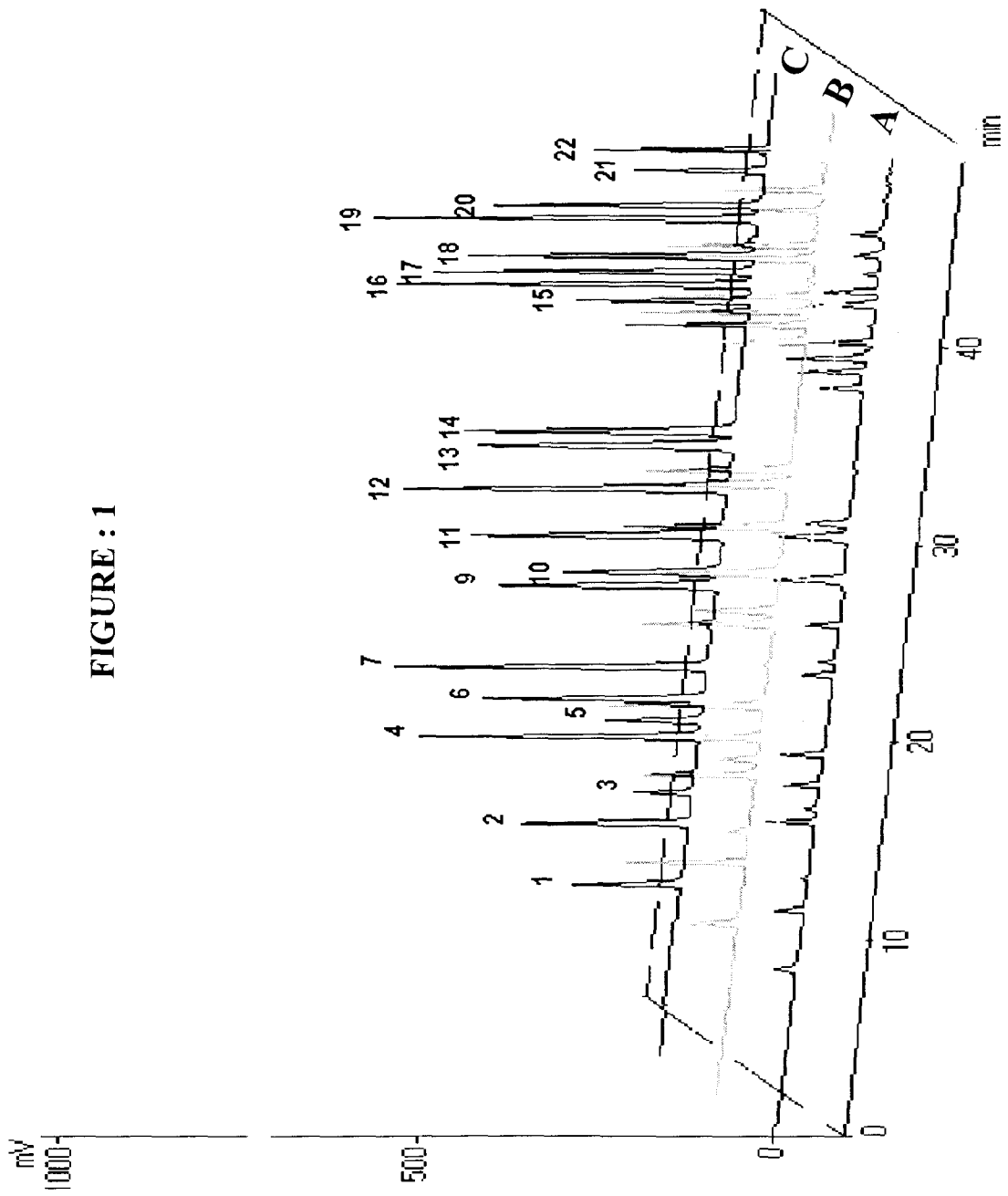
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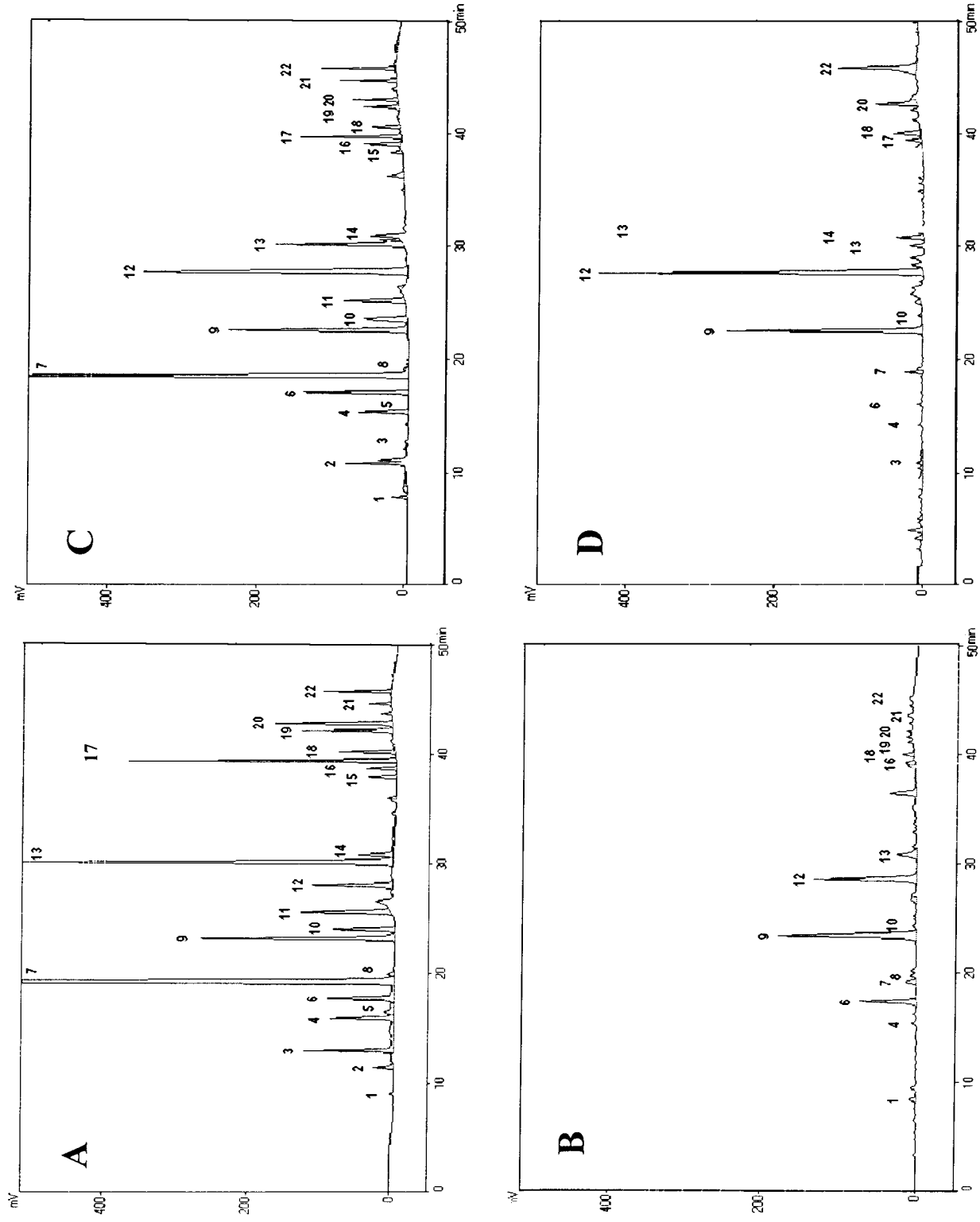
FIGURE : 1

FIGURE : 2



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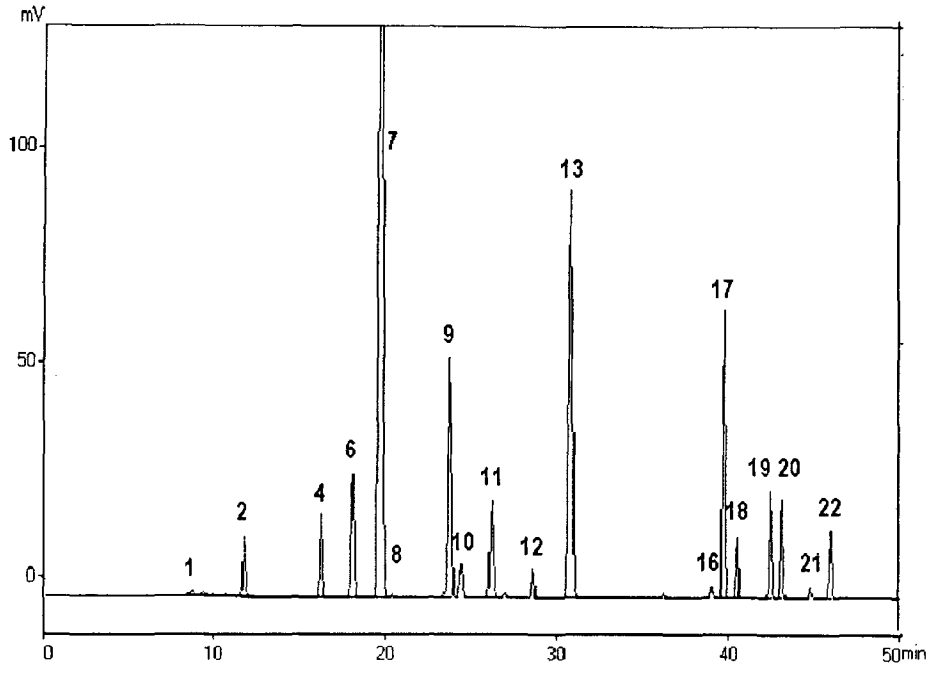
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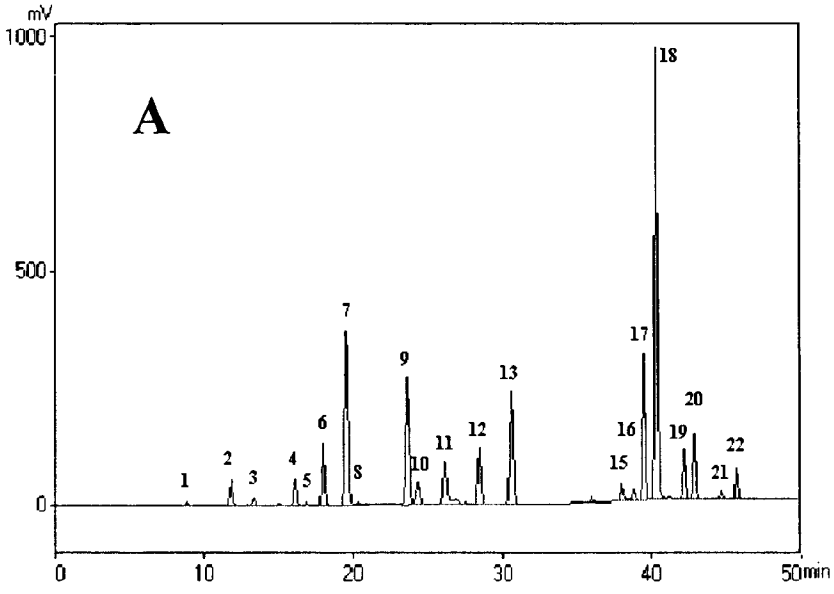
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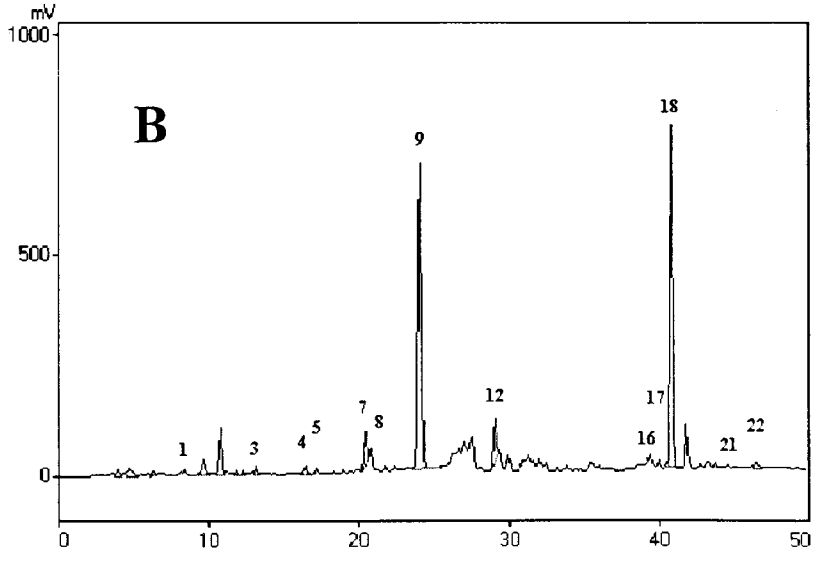
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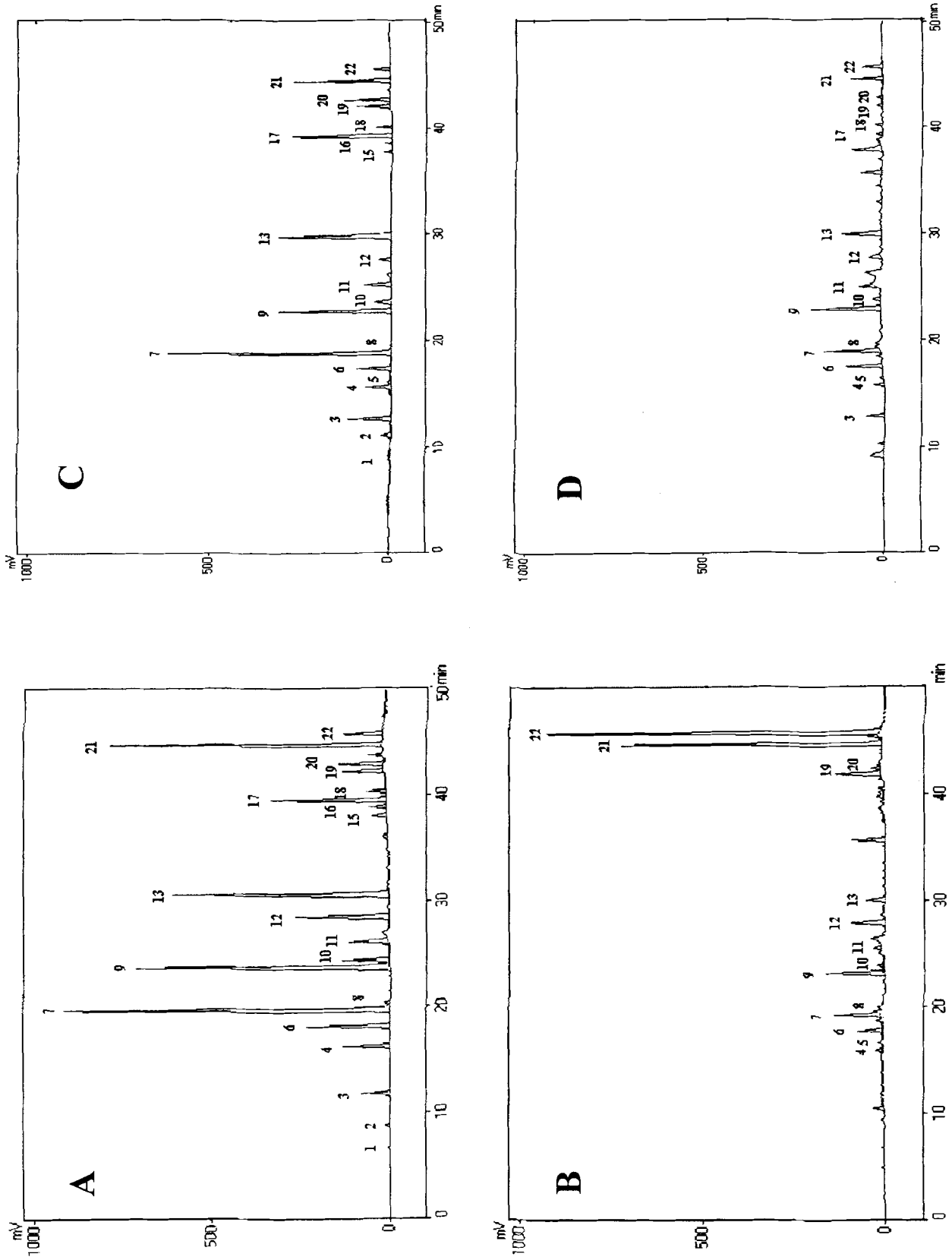
FIGURE: 5



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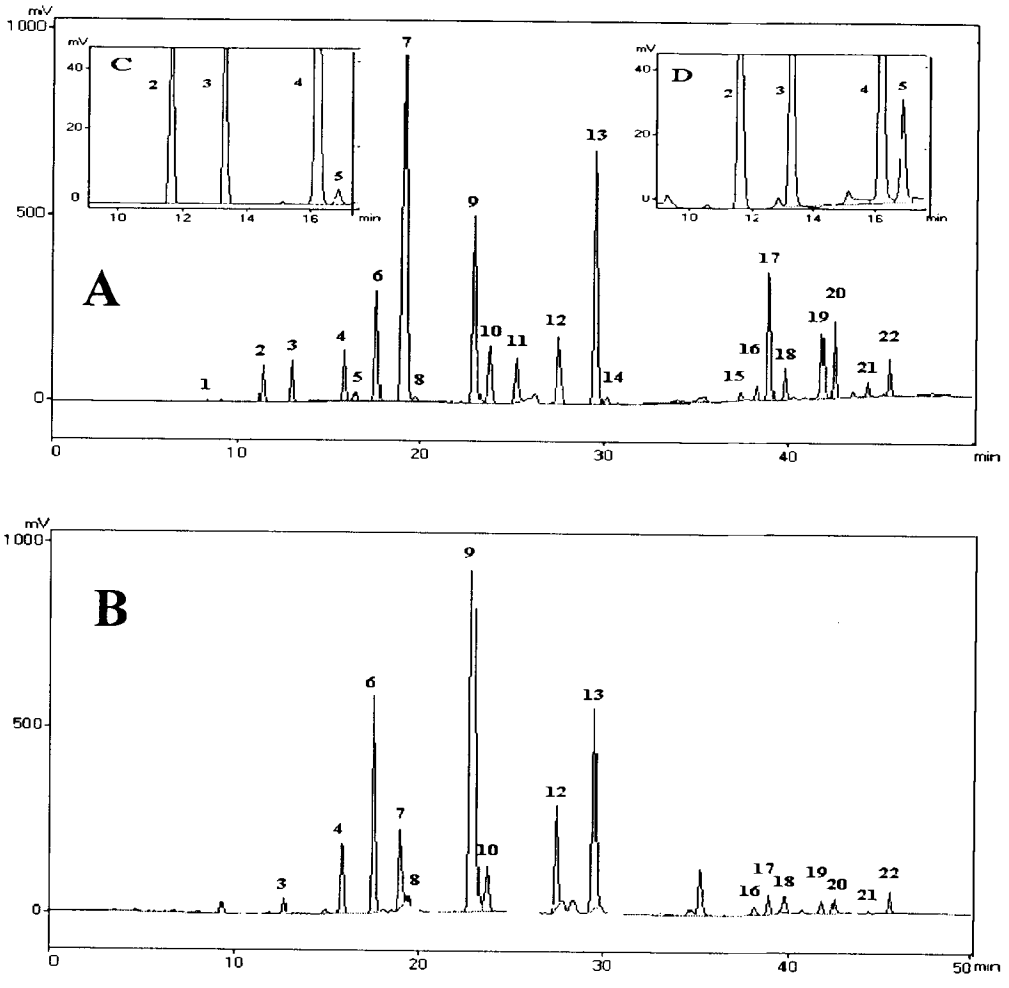

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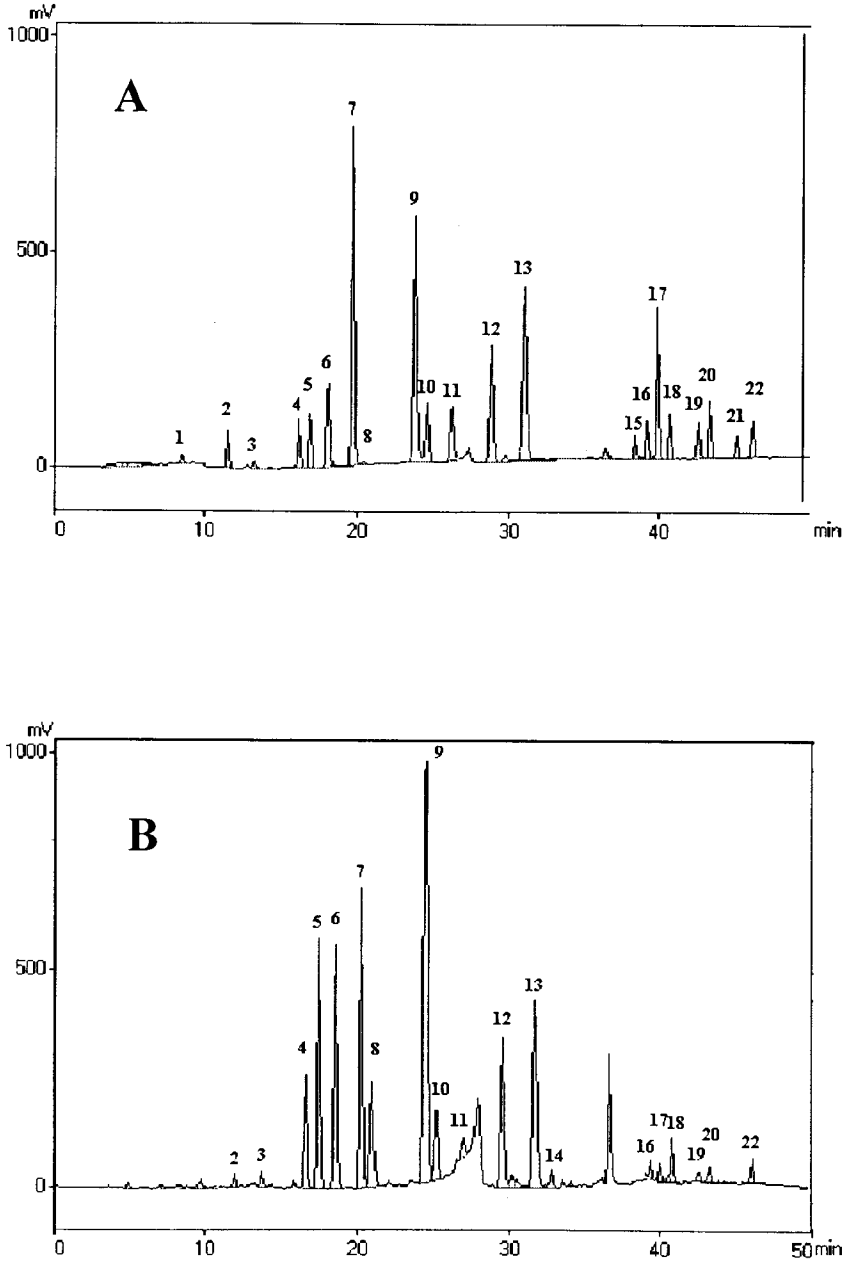
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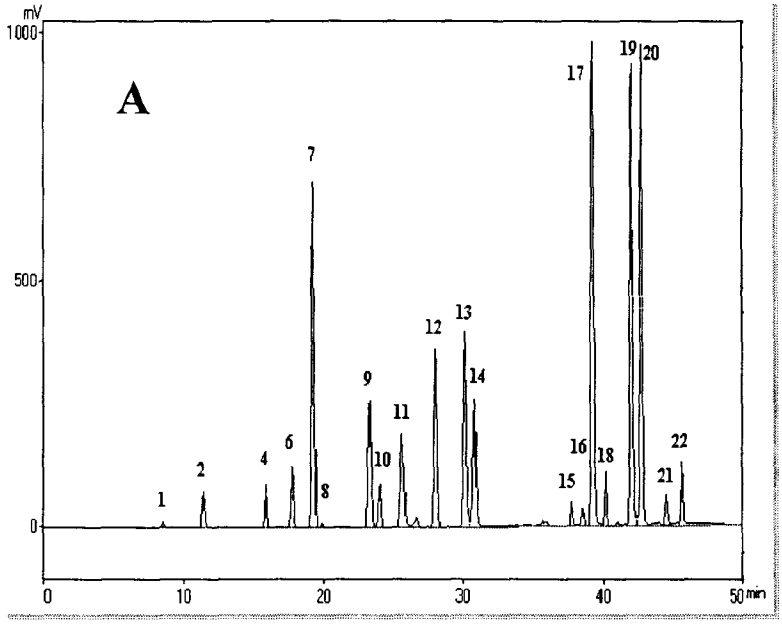
FIGURE : 8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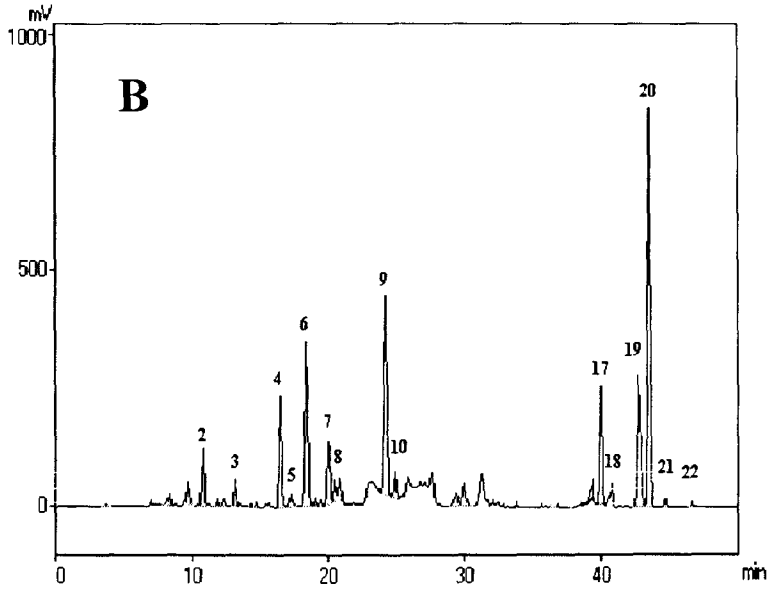
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FIGURE : 9

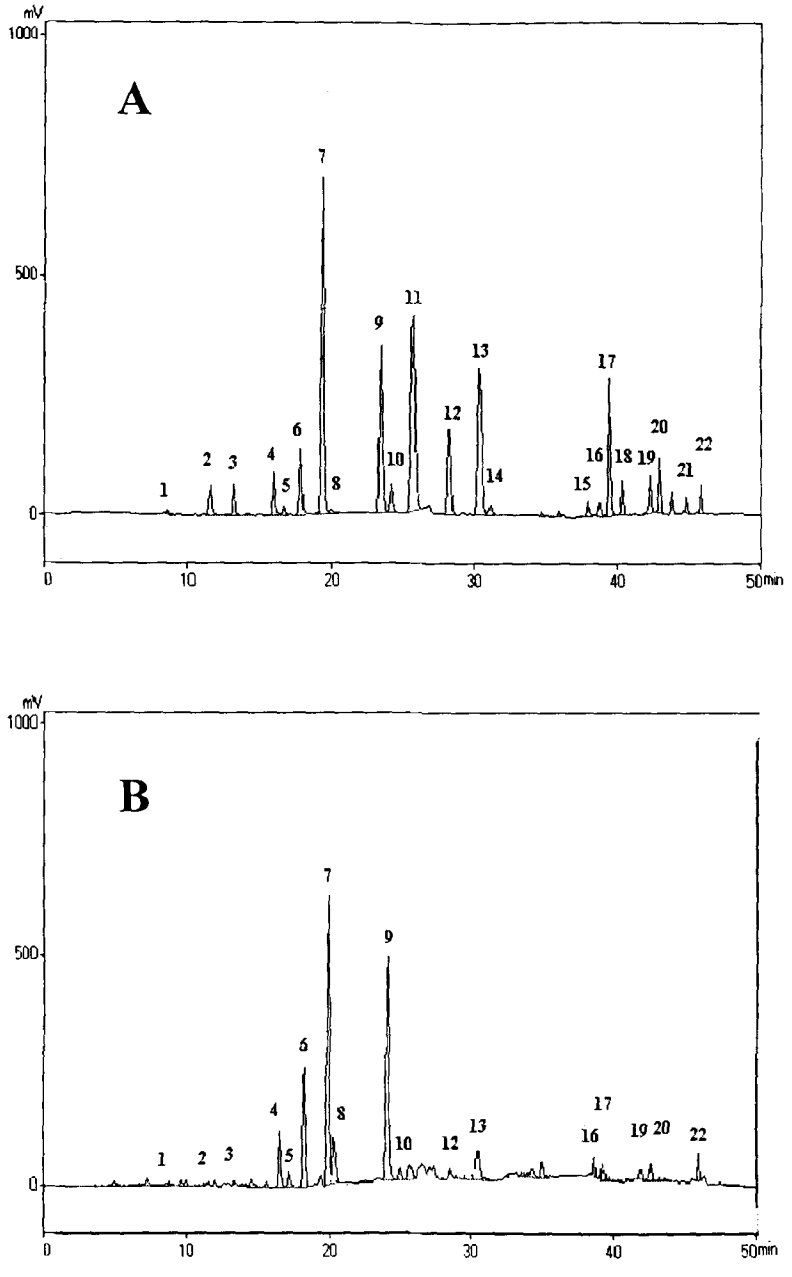
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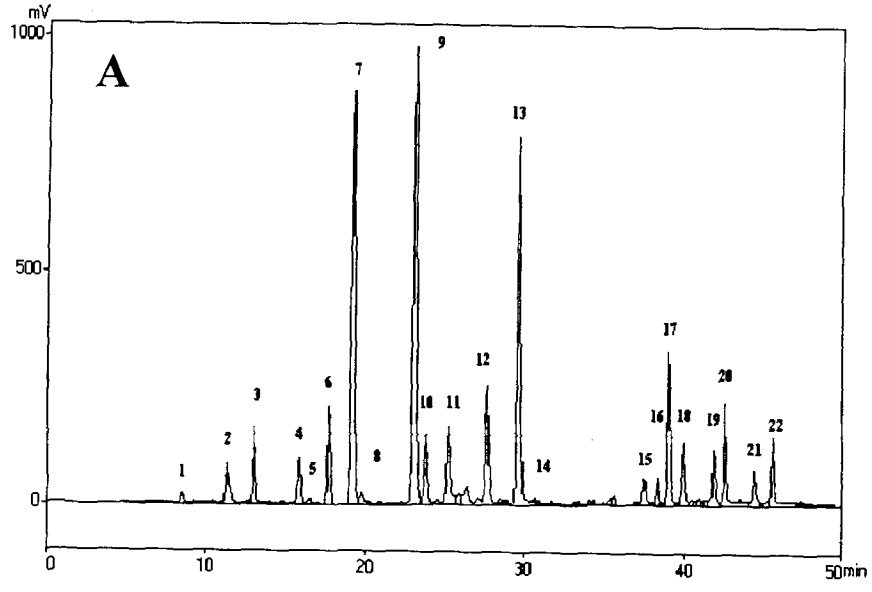
FIGURE : 10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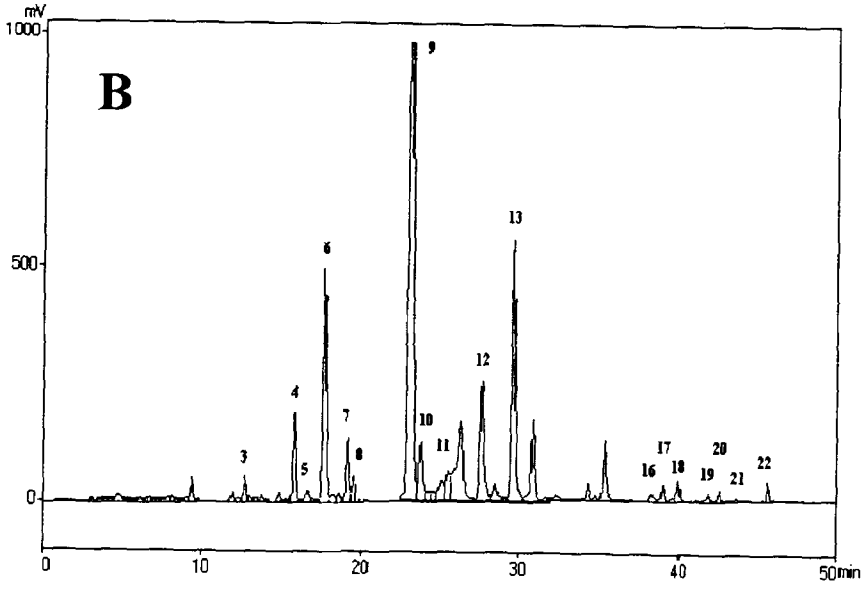
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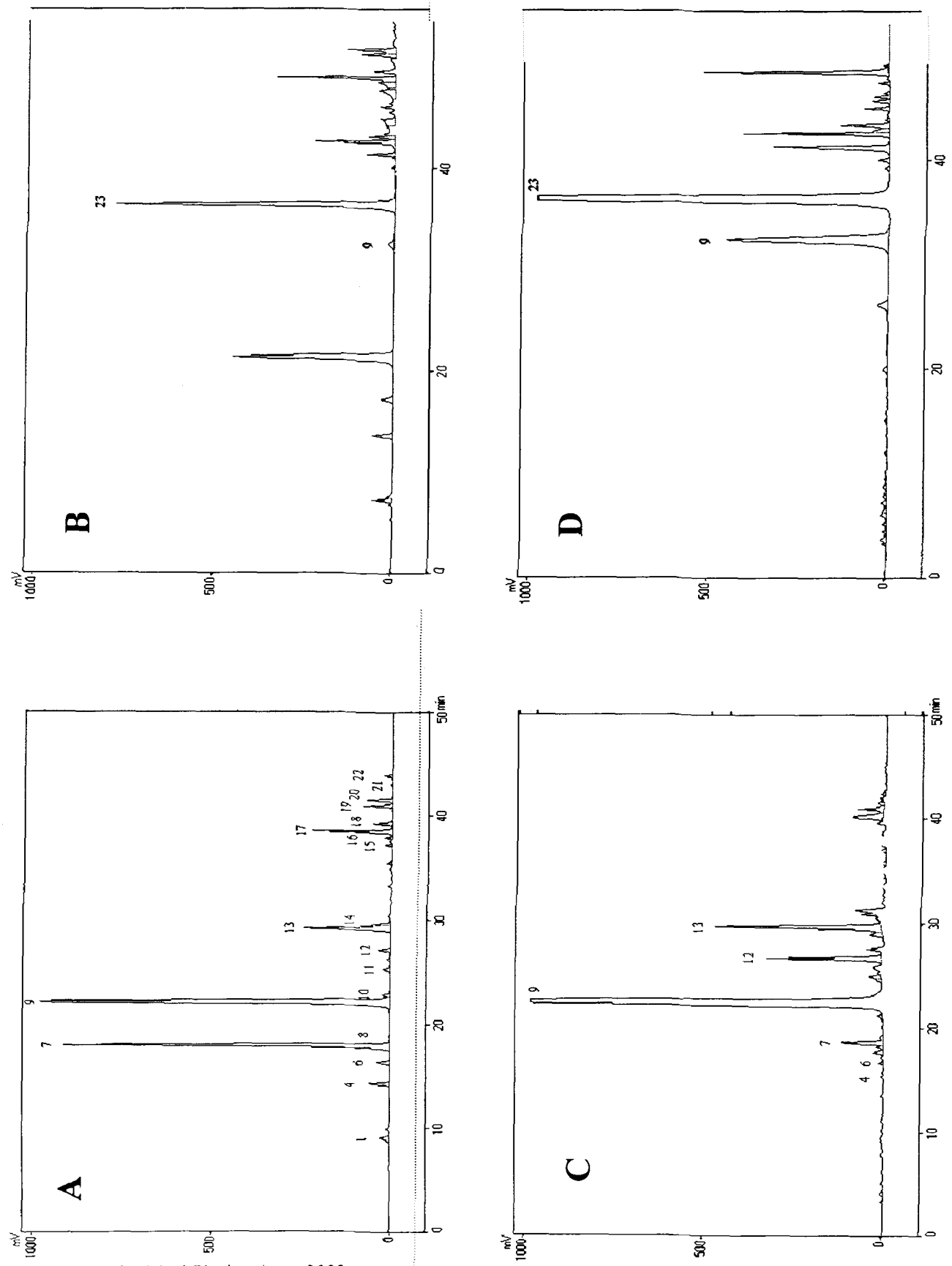
FIGURE : 11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