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A SIMPLE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NOMIFENSINE IN PLASMA AND A COMPARISON OF THE METHOD WITH OTHER AVAILABLE TECHNIQUES

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1 A rapid simple gas chromatographic technique for determining nomifensine in plasma is described.

2 Nomifensine is rapidly absorbed after oral administration to man.

3 Following a 100-mg dose, peak levels of an acid-labile conjugate of 2—3 μg/ml are reached about 1.5 h after administration.

4 The conjugate is cleared with a half-life of between 1 and 2 hours.

Introduction

In recent years, the monitoring of drugs in plasma following therapeutic doses has become more and more important. Not only does this give information on the kinetics of the drug, and therefore a greater appreciation of onset of action, dosage schedules, and so on, but a good analytical method is often a requirement for registration authorities. A number of methods have now been described in the literature for the measurement of nomifensine in plasma (Bailey *et al.*, 1977; Heptner *et al.*, 1977a; Vereczkey *et al.*, 1976; Uihlein & Hajdu, 1977). In this paper we describe a simple and rapid gas chromatographic technique for the determination of plasma levels of nomifensine. The results, using this method, are compared with those published by other workers.

Method for determination of nomifensine (free base) in plasma

To plasma (1 ml) is added 1 N NaOH (1 ml), internal standard, 8-amino-1,2,3,4-tetrahydro-2-butyl-4-phenyl isoquinoline (HOE49673) 400 ng or diazepam 500 ng in ethanol (10 μl) and freshly distilled peroxide-free ether (10 ml). The mixture is shaken gently in a Heto Rotamix shaker for 15 min and the layers allowed to separate. The ether layer is transferred to a conical tube and the solvent evaporated in a stream of nitrogen. The residue is thoroughly dried by standing in a vacuum desiccator for 1 hour. The dried residue is dissolved in ethanol (50 μl) and aliquots (5 μl) analyzed by gas chromatography.

Chromatography is on a Perkin-Elmer F-17 Gas Chromatograph equipped with a nitrogen-selective detector and a glass column (2 m) packed with 3% OV-25 on Chromosorb W-HP. At an oven temperature of 250°C and a carrier flow rate (helium) of 30 ml/min, nomifensine has a retention time of approximately 3 min and the internal standards have retention times of 4.5 min (HOE49673) and 6 min (diazepam). Peak heights of nomifensine and internal standard are measured and the ratio nomifensine peak height: internal standard peak height computed for each sample.

The concentration of nomifensine in the plasma is calculated from the corresponding ratio obtained by processing control plasma containing known amounts of nomifensine 100 and 200 ng/ml exactly as described above. A typical chromatogram obtained in the analysis of plasma from a normal volunteer dosed orally with nomifensine is shown in Figure 1.

Evaluation of method

Extraction of nomifensine

Nomifensine 500 ng in ethanol (10 μl) and diazepam 500 ng in ethanol (10 μl) were added to water (1 ml) and the solution processed as described in the standard method. Exactly 5 μl of the final 50 μl solution was chromatographed on 3% OV-17 at 240°C and the chromatogram compared with nomifensine 50 ng (injected as the maleate salt) and diazepam 50 ng chromatographed in the same conditions. The peak heights obtained after extraction were identical to those obtained by direct injection, indicating that both nomifensine and the internal standard, diazepam, were extracted quantitatively from water. A similar experiment was performed to demonstrate extraction of nomifensine from plasma. In this case, the peak height of nomifensine in the chromatogram obtained from the plasma extract was

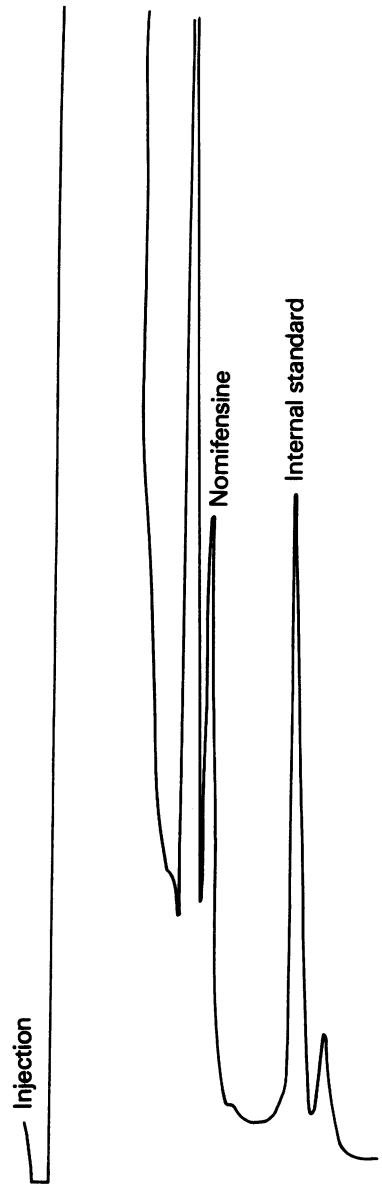

Figure 1 A typical gas chromatogram obtained in the analysis of plasma from a normal subject 2 h after receiving nomifensine 75 mg orally. The nomifensine corresponds to 45 ng/ml plasma. The internal standard was the nomifensine analogue, HOE49673.

identical to that obtained from the equivalent amount of standard, although the diazepam peak was slightly reduced. Thus, nomifensine seems to be quantitatively extracted after it has been added to the control plasma.

Choice of column

No extensive survey was carried out for the assay of nomifensine. Both OV-17 and OV-25 were used in the development of the method and, generally speaking, there was very little difference between the two. We have standardized on OV-25 for our method, as our present OV-25 column has slightly better efficiency than our OV-17 column.

Linearity of response as judged by peak height ratios

As only one or two standards are processed for each batch of samples, it was necessary to establish that the relative response ratio was constant over the expected range of analysis. Control human plasma, to which had been added known amounts of nomifensine, was processed using diazepam as the internal standard. The response was linear over the range 50—500 ng/ml.

Sensitivity

The sensitivity of the method is limited by the appearance of a small peak at the retention time of nomifensine which is seen in samples from control subjects. This peak corresponds to about 3 ng/ml nomifensine, and thus the sensitivity can be quoted as 10 ng/ml.

Specificity

Apart from the peak mentioned above, extracts of plasma and urine from control subjects do not contain any components with the retention time of nomifensine using our gas chromatographic conditions. Samples of known metabolites of nomifensine were also available (metabolites M_1, M_2 and M_3 described by Heptner *et al.*, 1977b); no peaks were observed, even after 20 min, when these three compounds were chromatographed on 3% OV-17 at 260°C.

Accuracy

Accuracy is defined as the closeness of the found value to the true value. In this assay, procedural losses and injection errors are automatically corrected by use of the internal standard. This, in conjunction with the specificity, ensures the accuracy of the method.

Stability of nomifensine in plasma extracts

Samples of human plasma to which had been added known amounts of nomifensine were processed in the usual way. The extracts of a series of samples were stored as the dried residue overnight; a second series was stored overnight in ethanolic solution. All

samples were chromatographed on 3% OV-25 at 260°C, both before and after storage. Overnight storage did not significantly alter the peak height ratios. There was, however, a noticeable effect on sensitivity if the samples are not analyzed immediately after processing. Thus, we recommend that samples are chromatographed as soon as possible after extraction.

Determination of total nomifensine

The method described is for the determination of free (that is, non-conjugated) nomifensine. A certain amount of nomifensine circulates and is excreted as an acid-labile conjugate (Heptner *et al.*, 1977b) and the total nomifensine can be determined after mild hydrolysis as follows.

To plasma (1 ml) is added 5 N HCl (100 μl). The sample tube is shaken with a vortex mixer and allowed to stand in the dark for 10 min. 1 N NaOH (1.5 ml) is added and the samples processed as described previously.

A series of human control plasmas containing known amounts of nomifensine were processed by the standard method for nomifensine and a parallel series were processed by the above method for total nomifensine.

A slight decrease (10%) in the values obtained using the modified method for total nomifensine indicated that there may be slight decomposition of the added nomifensine using the hydrolysis method.

Results and discussion

The described method was applied to the analysis of plasma obtained from three normal volunteers who had received nomifensine 75 mg orally. A fourth subject received 50 mg by the same route. The results obtained without acid hydrolysis are summarized in Table 1. Other available methods are summarized in Table 2. Full details of these methods are described in published papers.

Following an oral dose of 50 mg, Vereczkey *et al.* (1976) reported peak plasma levels at 1.5 h of 120 ng/ml. Following an oral dose of 75 mg, Bailey *et al.* (1977) reported similar values, that is, 130 ng/ml at 1–2 h after the dose. These peak levels were considerably higher than those reported here and the values more recently reported by Uihlein & Hajdu (1977); 20–50 ng/ml after 100 mg and by Heptner *et al.* (1977a); 53 ng/ml after 100 mg.

To further evaluate our simple method, the plasma samples from three subjects dosed with nomifensine 75 mg were further analyzed in the laboratories of Dr Uihlein and Dr Heptner, using high pressure liquid chromatography and radioimmunoassay, respectivly. All three centres found similar levels of nomifensine in the plasma of these subjects (Table 1). The radioimmunoassay results, however, were obtained after a correction had been made to account for cross-reactivity of the conjugate, thus, the accuracy of the values reported may be suspect. The gas chromatographic methods described by Bailey *et al.* (1977) and by Vereczkey *et al.* (1976) used longer extraction procedures than those either in the present method or by Uihlein & Hajdu (1977).

The labile conjugate may have been extracted and converted to nomifensine by a subsequent back extraction into hydrochloric acid. This was investigated by extracting plasma from a dosed volunteer several times and then analyzing each ether extract for nomifensine by the method of Bailey *et al.* (1977). Nomifensine was found only in the first extract, indicating that partial extraction of a conjugate would not explain the high levels of nomifensine found. We conclude that the most likely explanation is that there had been some degradation of the conjugate to nomifensine before analysis and

Table 1 Plasma concentrations (ng/ml) of nomifensine in normal subjects receiving nomifensine 75 mg orally

Time after dose	*Subject 1*			*Subject 2*			*Subject 3*		
	Method 1	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*
0	0	0	0	0	0	0	0	0	0
0.5	42	43	66	<10	<10	—	<10	42	—
1	46	54	72	<10	38	12	14	57	28
1.5	16	47	48	14	41	48	10	53	45
2	20	45	26	<10	—	19	10	43	34
3	14	33	6	<10	32	14	56	<10	—
5	<10	<10	4	<10	<10	4	10	<10	—
8	<10	<10	—	<10	<10	—	<10	<10	—

Nomifensine was determined by three different methods: (1) present method; (2) high pressure liquid chromatography (Uihlein & Hajdu, 1977); (3) radioimmunoassay (Heptner *et al.*, 1977a).

Table 2 Summary of methods available for the determination of nomifensine in plasma or serum

Reference	*Method*	*Accuracy*	*Sensitivity*	*Specificity*	*Practicality (number of assays per day)*
Vereczkey *et al.* (1976)	GC of heptafluorobutyrate with EO detector	Recovery 93 ± 2%	1–5 ng/ml	Chromatographic method	
Present method	GC of free base with NS detector	Reference to standards taken through the method with internal standard	10 ng/ml	Chromatographic method	30
Bailey *et al.* (1977)	GC of heptafluorobutyrate with NS detector	Reference to standards taken through the procedure with two internal standards ± 5.4%	2 ng/ml	Chromatographic method. Identity of peak confirmed by mass spectrometry	36
Uihlein & Hajdu (1977)	HPLC of free base	Reference to standards taken through the method with an internal standard	10 ng/ml	Chromatographic method. Identity of peak confirmed by mass spectrometry	25
Heptner *et al.* (1977a)	RIA	Reference to standard curve prepared from admixed serum	1 ng/ml	Does not cross react with known metabolites, but does cross react with conjugates	250

GC, Gas chromatography; HPLC, high pressure liquid chromatography; RIA, radioimmunoassay

Table 3 Plasma concentration (μg/ml) of nomifensine in normal subjects receiving nomifensine 75mg orally

Time after dose	*Subject 1*			*ug/ml Subject 2*			*Subject 3*			*Subject 4*
	Method 1	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*	*Method 4*
0	0	0	0	0	0	0	0	0	0	—
0.5	2.58	1.76	2.32	0.21	0.19	0.28	0.89	0.96	1.76	0.04
1	2.04	—	2.86	0.90	0.93	1.62	2.20	1.85	2.74	0.54
1.5	0.87	1.78	2.15	1.33	2.09	2.16	2.32	1.80	2.16	1.03
2	0.61	1.09	1.68	0.90	—	1.80	1.56	1.09	1.47	1.15
3	0.48	0.53	1.12	0.26	0.45	0.71	0.27	0.49	1.02	0.44
5	0.11	0.26	0.42	0.08	0.16	0.24	0.15	0.25	0.38	0.31
7	—	—	—	—	—	—	—	—	—	0.19
8	0.02	0.13	0.22	0.06	0.04	0.08	0.02	0.14	0.18	—
9	—	—	—	—	—	—	—	—	—	0.04

All samples were treated with hydrochloric acid before analysis.

Methods: (1) present method; (2) high pressure liquid chromatography (Uihlein & Hajdu, 1977); (3) radioimmunoassay (Heptner *et al.*, 1977a); (4) gas chromatography (Bailey *et al.*, 1977).

that careful sampling and storage is necessary when carrying out nomifensine plasma level studies.

Our results after acid hydrolysis are summarized in Table 3. The results obtained from a further normal volunteer, after hydrolysis of plasma, using the method of Bailey *et al.* (1977) are included in this Table for comparison. It can be seen that, following hydrolysis of the labile conjugate, considerably higher levels of nomifensine are measured by all techniques.

We conclude from the present study and from the comparisons made with previous studies that nomifensine is rapidly absorbed after oral administration to humans. Following a dose of 100 mg the peak plasma levels of nomifensine, mainly in the form of an acid-labile conjugate, are 2–3 μg/ml and are reached approximately 1.5 h after dosage. The conjugate is cleared with a half-life of 1–2 hours. The amount of unmetabolized nomifensine in the plasma is uncertain but is probably less than 50 ng/ml at the peak following an oral dose of 100 mg. It may even be argued that the low levels seen by the present method, and by Uihlein & Hajdu (1977) using high pressure liquid chromatography, are due to conversion of the labile conjugate to nomifensine subsequent to collection of the sample.

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