Journal of Chromatography, 490 (1989) 464–469
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO 4689

Note

Assay of ambroxol in human plasma by high-performance liquid chromatography with amperometric detection

F.J. FLORES-MURRIETA*, C. HOYO-VADILLO, E. HONG and G CASTAÑEDA-HERNANDEZ

Sección de Terapéutica Experimental, Departamento de Farmacología y Toxicología, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 22026, 14000 Mexico, D.F (Mexico)

(First received September 15th, 1988; revised manuscript received January 12th, 1989)

Ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride, is a bromhexine derivative with potent mucolytic activity widely used as an expectorant agent in therapeutics [1]. At present, only limited information is available concerning the determination of ambroxol in biological fluids. Vergin et al. [2] and Botterblom et al. [3] described high-performance liquid chromatographic (HPLC) methods with UV detection. However, these methods are not satisfactory for biomedical work, as no internal standard is used. Schmid [4] reported a capillary gas chromatographic method with electron-capture detection using an ambroxol derivative as internal standard. Although this method is suitable for pharmacokinetic studies, it requires a time-consuming derivatization step.

In recent years, HPLC with electrochemical detection has come into widespread use for the determination of easily oxidizable or reducible organic compounds [5]. As the ambroxol molecule bears several oxidizable groups, we developed a method using this technique for its rapid determination in plasma samples with appropriate precision and detection limit using pelanserin, a quinazolinedione derivative [6], as internal standard. The method was applied to the determination of ambroxol levels in healthy volunteers after solution and tablet administration.

0378-4347/89/$03.50

EXPERIMENTAL

Chemicals and solutions

Ambroxol was obtained from Laboratorios Promeco de Mexico (Mexico City, Mexico) and pelanserin, the internal standard, from the Sección de Terapéutica Experimental, Centro de Investigación y de Estudios Avanzados del IPN (Mexico City, Mexico). Methanol was of chromatographic grade (E. Merck, Darmstadt, F.R.G.). Deionized water was prepared using a Milli-Q system (Continental Water Systems, El Paso, TX, U.S.A.). All other reagents were of analytical-reagent grade.

Stock solutions of ambroxol and of the internal standard corresponding to a free base concentration of 0.912 mg/ml were prepared in deionized water. Standard solutions were prepared by diluting the stock solution to free base concentrations ranging from 91.2 to 1368 ng/ml in deionized water. A standard solution of the internal standard was prepared at a fixed free base concentration of 182.4 ng/ml. Sodium borate (0.025 *M*), providing a pH of 9.0, and hydrochloric acid (0.1 *M*) were prepared in deionized water.

Sample preparation

Blood was collected from healthy subjects by means of an indwelling cannula with a heparin lock placed in a suitable forearm vein. Plasma was obtained by centrifugation at 3000 *g* for 15 min. Plasma specimens (1 ml) were pipetted into conical glass tubes and spiked with 0.1 ml of a 182.4 ng/ml solution of internal standard. After addition of 1 ml of 0.025 *M* sodium borate solution, the alkalinized plasma was extracted with 3 ml of diethyl ether using a Yankee variable-speed rotator (Clay Adams, Parsippany, NJ, U.S.A.) set at 150 rpm for 15 min. The two phases were separated by centrifugation at 3000 *g* for 5 min. The upper organic layer was transferred into another conical glass tube and the plasma was extracted again with 3 ml of diethyl ether. The combined organic layers were evaporated to dryness at 40 °C under a stream of nitrogen. The dry residue was reconstituted with 0.5 ml of *n*-heptane and a back-extraction step with 0.5 ml of 0.1 *M* hydrochloric acid was performed. The two phases were separated by centrifugation, as described above, and the organic layer was discarded. Aliquots of the aqueous layer (0.2 ml) were injected into the chromatographic system.

Chromatographic conditions

The chromatographic system consisted of a Model 510 solvent-delivery system (Waters Assoc., Milford, MA, U.S.A.), a Model LC-22A temperature controller (BAS, West Lafayette, IN, U.S.A.), an electrochemical transducer coupled to a Model LC-4B amperometric detector (BAS) and a 200-μl loop injector (Rheodyne, Cotati, CA, U.S.A.). Analyses were performed on a 300 mm×4 mm I.D. MCH-10 column (Varian, Palo Alto, CA, U.S.A.) using methanol–0.1

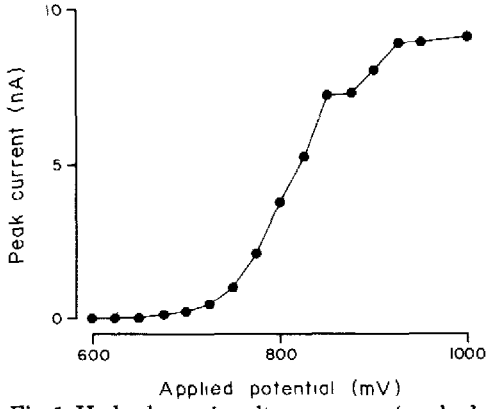

Fig. 1. Hydrodynamic voltammogram (applied potential versus output current) of ambroxol. Output current was determined by injecting samples of the same concentration (100 ng in 0.2 ml) at various potentials.

M phosphate buffer (pH 6.5) (65:35, v/v) as the mobile phase. The column was kept at 40 °C and flow-rate was kept constant at 1.5 ml/min. As there was no information available concerning the electrochemical properties of ambroxol, the peak current was recorded at several applied potentials (Fig. 1). It appeared that 850 mV was the most suitable potential as a good response was obtained, maintaining a high selectivity. Current was detected at a sensitivity of either 2 or 5 nA full scale, depending on the amount of ambroxol injected into the system. To prolong the life of the analytical column, a precolumn (35 mm×4 mm I.D.) containing 37–50 μm Corasil C_{18} (Waters Assoc.) was incorporated in the system.

Calibration

The assay was calibrated by addition of known amounts of ambroxol and internal standard to drug-free plasma samples (1 ml). Calibration graphs were established for ambroxol free base concentrations ranging from 9.12 to 136.8 ng/ml. The internal standard was used at a fixed free base concentration of 18.24 ng/ml. The ultimate sample concentrations were calculated by determination of the peak-height ratios of ambroxol to the internal standard.

RESULTS

Typical chromatograms of extracted plasma samples are shown in Fig. 2. The retention times for the internal standard and ambroxol were 8.4 and 11.4 min, respectively. No interfering peaks occurred at these times. Any endogenous contaminants remaining in the extracts were eluted before the internal standard, and samples could be injected immediately after elution of ambroxol. A linear relationship ($r=0.997$) was found when the ratio of the peak height

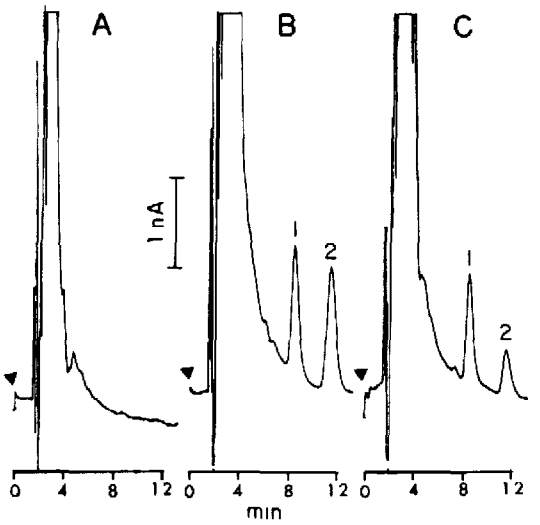

Fig. 2. Chromatograms of human plasma extracts. (A) Drug-free plasma; (B) spiked plasma containing (1) 18.24 ng/ml internal standard and (2) 45.6 ng/ml ambroxol; (C) plasma of a healthy volunteer 2.5 h after a 30-mg oral dose spiked with 18.24 ng/ml internal standard.

TABLE I

ACCURACY AND COEFFICIENT OF VARIATION OF THE HPLC METHOD FOR DETERMINATION OF AMBROXOL IN PLASMA SAMPLES

Theoretical concentration (ng/ml)	Observed concentration (mean ± S.E.M.[a], $n=5$) (ng/ml)	Accuracy (%)	Coefficient of variation (%)
9.12	8.43 ± 0.05	92.4	1.12
18.24	17.33 ± 1.59	95.0	8.63
45.6	44.6 ± 1.03	97.8	5.28
91.2	97.64 ± 1.60	107.1	3.31
135.5	131.67 ± 3.56	96.3	5.46

[a]Standard error of the mean.

of ambroxol to that of the internal standard was plotted on the ordinate against various concentrations of ambroxol (free base) ranging from 9.12 to 136.8 ng/ml. The equation by the least-squares method was $y=0.02315x-0.038$.

The recoveries of ambroxol and the internal standard from plasma samples were similar and ranged from 80 to 90%, by comparison of peak heights from plasma extracts with those from standard solutions. The accuracy and precision of the assay were ascertained by adding known amounts of ambroxol to drug-free plasma and analysing the samples. The results are summarized in

TABLE II

PLASMA LEVELS OF AMBROXOL AFTER ORAL ADMINISTRATION OF 30 mg AS A SOLUTION OR AS A TABLET IN SIX HEALTHY VOLUNTEERS (MEAN ± S.E.M.)

Time (h)	Plasma level (ng/ml)	
	Solution	Tablet
1.0	35 ± 14	33 ± 7
1.5	59 ± 13	38 ± 12
2.0	47 ± 12	37 ± 11
2.5	37 ± 10	38 ± 8
8.0	22 ± 4	20 ± 5
12.0	17 ± 4	15 ± 5

Table I. The intra-assay precision was determined over a period of four weeks and the coefficients of variation were 7.1% at 18.24 ng/ml ($n=7$) and 5.4% at 91.2 ng/ml ($n=7$). The detection limit (signal-to-noise ratio=2) was 1 ng/ml.

The effectiveness of the method for pharmacokinetic and bioavailability studies was examined by measuring plasma levels in six healthy volunteers after oral administration of ambroxol. Each subject received 10 ml of a 3 mg/ml solution and one 30-mg tablet in a crossover design. The interval between medications was one week. Blood samples were drawn and ambroxol plasma concentrations were determined by the procedure described. The results are given in Table II.

DISCUSSION

Pelanserin, a quinazolinedione derivative [6], was used as the internal standard as it is extracted with diethyl ether, separated by reversed-phase HPLC and yields a high-current response at 850 mV, the optimum potential for detection of ambroxol. The method has been demonstrated to be sufficiently sensitive and precise for the determination of ambroxol plasma levels in the concentration range required for pharmacokinetic and bioavailability studies. The entire procedure can be easily carried out by a single person and, as injections can be made every 13 min, over thirty samples can be assayed daily.

No time-consuming derivatization step is required with our procedure, as in the methods described by Vergin et al. [2] and by Schmid [4]. Plasma concentrations observed after a 30-mg oral administration of ambroxol, in either solution or tablet form, were similar to those reported by these workers. A very rapid HPLC method using UV detection was published recently [3]. However, although a detection limit of 5 ng/ml was claimed, the accuracy and precision were only evaluated at plasma concentrations above 30 ng/ml, whereas the

levels found after the usual 30-mg oral dose [4] are frequently lower, as can be seen in Table II.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. J.A. Rojas and O.A. Chassin for providing plasma samples from healthy volunteers dosed with ambroxol, Miss H. Garduño and Mrs. A. Herrera for technical assistance and Mr. A. Franco for drawings. This work was supported by COSNET-SEP PEP 04.87 and Laboratorios Promeco de México. F.J. Flores-Murrieta is a CONACYT fellow.

REFERENCES

1 S. Püschman and R. Engelhorn, Arzneim.-Forsch., 29 (1978) 889.
2 H. Vergin, G.B. Bishop-Freudling, M. Miczka, V. Nitsche, K. Strobel and F. Matzkies, Arzneim.-Forsch., 35 (1985) 1591.
3 M.H.A. Botterblom, T.J. Janssen, P.J.M. Guelen and T.T. Vree, J. Chromatogr., 421 (1987) 211.
4 J. Schmid, J. Chromatogr., 414 (1987) 65.
5 P.T. Kissinger, in P.T. Kissinger and W.R. Heineman (Editors), Laboratory Techniques in Electroanalytical Chemistry, Marcel Dekker, New York, Basle, 1984, Ch. 22, pp. 611–635.
6 F.J. Flores-Murrieta, E. Hong and G. Castañeda-Hernández, J. Chromatogr., 428 (1988) 167.