Journal of Chromatography, 421 (1987) 216–222
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3805

Note

Applications of column-switching techniques in biopharmaceutical analysis

II. High-performance liquid chromatographic determination of tripelennamine in bovine plasma and milk

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(First received March 3rd, 1987; revised manuscript received May 28th, 1987)

Tripelennamine, 2-[benzyl(2-dimethylaminoethyl)amino]pyridine (Fig. 1), is an antihistamine which acts as a competitive antagonist of histamine at H_1 receptors [1]. Tripelennamine is used to control allergic manifestations in humans and animals and it has also been used as a stimulant in cows who refuse to stand because of various disorders [2].

The chromatographic methods employed for the determination/detection of tripelennamine include thin-layer chromatography [3–5] to detect tripelennamine in urine when screening for drug abuse and gas chromatography (GC) with flame ionisation detection for the identification of the drug in urine [3,5], to determine tripelennamine residues in bovine milk [2] and in pharmaceutical formulations [6]. GC has also been used with nitrogen-specific detection [7,8] to determine tripelennamine in the blood of addicts taking a combination of pentazocine and tripelennamine known as T's and Blues. High-performance liquid chromatographic (HPLC) methods have been reported for the determination of basic antihistamines including tripelennamine [9] and in animal feed, human urine and waste water [10].

All the above methods either lack sufficient sensitivity or a lengthy and tedious extraction step is required prior to chromatography. The objective of the present study was to develop an HPLC method for the determination of tripelennamine in bovine plasma and milk that is sensitive, selective and reproducible, and yet convenient in terms of sample handling and speed of analysis. In the following sections we describe a technique which fully meets the objectives set out above.

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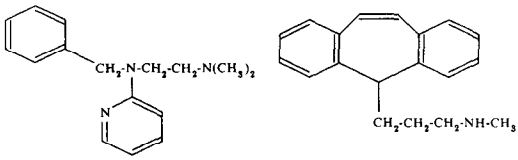

Tripelennamine Protriptyline

Fig. 1. Chemical structures of tripelennamine and protriptyline.

EXPERIMENTAL

Reagents and solvents

Tripelennamine hydrochloride was obtained from Sigma (Poole, U.K.). Protriptyline hydrochloride, internal standard, was obtained as a gift from Jervis Street Hospital (Dublin, Ireland). Methanol and acetonitrile (HPLC grade) were purchased from Fisons (Loughborough, U.K.) and sodium acetate (Analar grade) was purchased from BDH (Poole, U.K.). Pure water was obtained by the Milli-Q water purification system. Drug-free bovine plasma and milk were provided by the Bimeda Chemicals (Dublin, Ireland), stored frozen at $-4°C$ and thawed at room temperature prior to use. The control plasma and milk showed no interference for endogenous components when examined for possible interferences with the tripelennamine assay system.

Preparation of standards

Tripelennamine hydrochloride (11.43 mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml. This stock solution was then diluted with methanol–water (1:1) to yield working standards in the concentration range 0.04–6 μg/ml. Protriptyline hydrochloride (11.39 mg) was weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml. This was then diluted with methanol–water (1:1) to obtain a working internal standard solution of 4 μg/ml. Spiked plasma samples ranging in concentration from 2 to 50 ng/ml of the drug in plasma were prepared in each assay day by spiking 1 ml of plasma with 50 μl of working standards (0.04–1 μg/ml) and 50 μl of internal standard (4 μg/ml) solution. Spiked milk samples ranging from 5 to 300 ng/ml of the drug in milk were prepared by spiking 1 ml of milk with 50 μl of working standards (0.1–6 μg/ml) and 50 μl internal standard (4 μg/ml) solution.

Samples from cattle undergoing treatment were spiked with 50 μl of methanol–water (1:1) to compensate for any changes in the composition of the sample plus 50 μl of internal standard (4 μg/ml).

Instrumentation and chromatography

The HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) P-45 liquid chromatograph solvent delivery systems equipped with a Waters U6K manual injector and a Rheodyne (Cotati, CA, U.S.A.) 7000, six-port switching valve. A Shimadzu (Tokyo, Japan) SPD-6A variable-wavelength UV detector

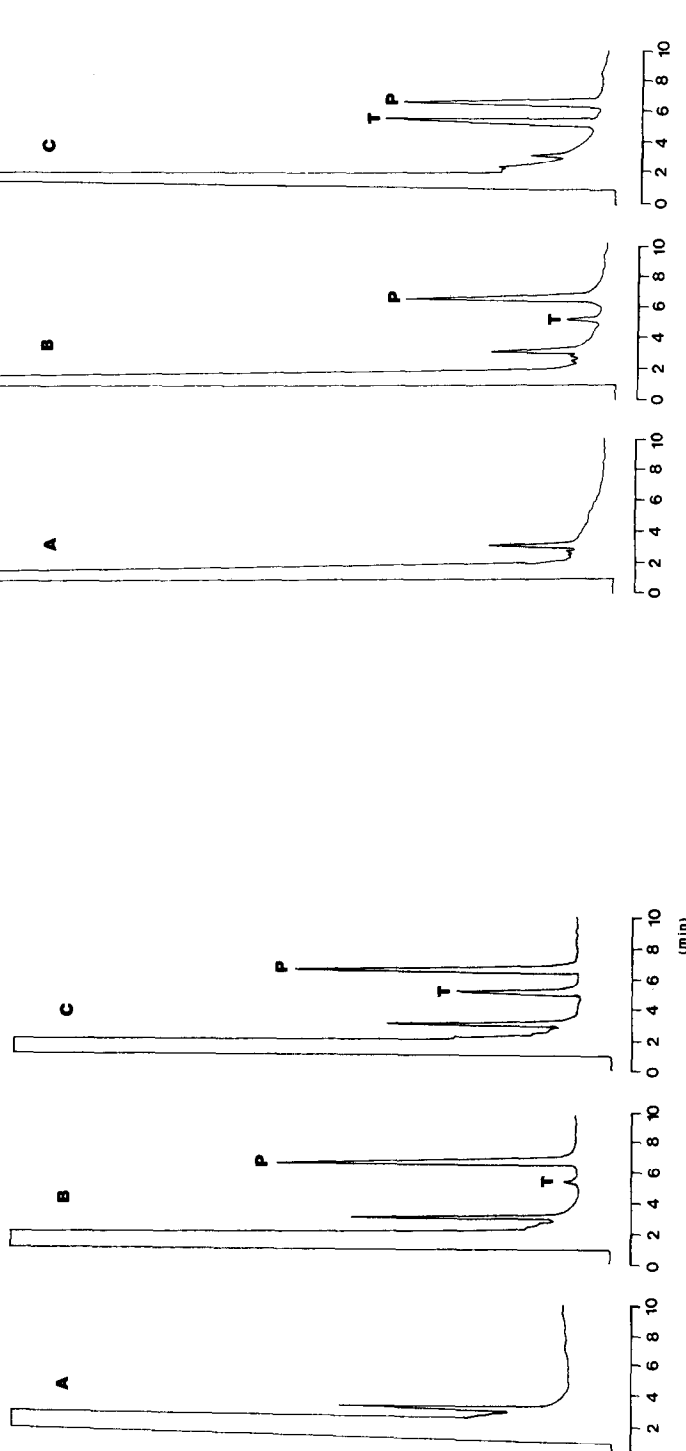

Fig. 2. (Left) Chromatograms of (A) a small pooled bovine drug-free plasma, (B) drug-free plasma spiked with internal standard and 2 ng/ml tripelennamine and (C) plasma of a cattle 30 min after receiving an intravenous injection of a formula containing tripelennamine. (Right) Chromatograms of (A) a pooled bovine drug-free milk, (B) drug-free milk spiked with internal standard and 5 ng/ml tripelennamine and (C) milk of a cattle 30 min after receiving an intravenous injection of a formula containing tripelennamine. Peaks: T = tripelennamine; P = protriptyline.

was used and the chromatograms were recorded on a Philips (Eindhoven, The Netherlands) PM8251 single-pen recorder.

The instrument arrangement for the chromatography was exactly as described in our previous publication [11]. The chromatographic conditions for the separation were as follows: mobile phase pump A, water; concentration column (10×1.5 mm), dry packed in house with Corasil (Waters Assoc.) RP 18, 37–50 μm packing. The mobile phase from pump A was passed through the concentration column for 3 min (wash time) at a flow-rate of 0.8 ml/min. Mobile phase pump B, acetonitrile–0.05 *M* acetate buffer adjusted to pH 7.2 with acetic acid (70:30, v/v); analytical column, Techsphere (HPLC Technology, Macclesfield, U.K.) 3CN (10 cm×4.6 mm); flow-rate, 0.9 ml/min; detection wavelength, 246 nm; injection volume, 250 μl of plasma or milk sample.

Under the described chromatographic conditions the mean retention times were 5.2 and 6.8 min for tripelennamine and protriptyline, respectively (Fig. 2).

Column-switching procedure

The column-switching procedure described by Dadgar and Power [11] was adopted for use in the present study. The bovine plasma was more viscous than human plasma and the wash time was therefore extended to 3.0 min per sample to avoid clogging of the pre-column and the resulting high back-pressure. It was possible to inject up to thirty samples per concentration column. Chromatography deteriorated where sample injections exceeded thirty. Because of high concentration of fat in milk, it was not possible to directly inject the milk samples. Spinning the milk samples in a centrifuge at ca. 1200 *g* to separate fat from the sample proved to be as effective as extraction of fat in hexane. Upon centrifugation of milk samples, three distinct layers were formed. The top layer was the fat and the bottom layer was the precipitated protein. Hence, care should be exercised to inject the middle layer for chromatography. The analysis of separated fat and protein showed no detectable amount of tripelennamine (at a spiked level of 300 ng/ml tripelennamine in milk) in either fat or protein.

Calibration and calculations

Evaluation of the assay was carried out using five-point calibration standards in the concentration range 2–50 ng/ml tripelennamine in plasma and six-point calibration standards in the concentration range 5–300 ng/ml of the drug in milk. The slope and intercept of the calibration curves were obtained by linear regression of the peak-height ratios of drug/internal standard versus the concentration of the drug (internal standard method). These calibration curves were then used to interpolate the concentration of tripelennamine in bovine plasma and milk from the measured peak-height ratios.

RESULTS AND DISCUSSION

Limit of detection

Under the described procedural conditions, the limit of detection using a 250-μl direct injection of plasma or milk was 2 ng/ml tripelennamine in the biological

TABLE I

PRECISION (REPEATABILITY AND REPRODUCIBILITY) ($n=4$)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Difference between added and found concentration (%)
Intra-assay (repeatability)			
2	1.90 ± 0.19	9.7	5.0
5	4.90 ± 0.33	6.8	2.2
10	10.30 ± 0.25	2.5	3.0
25	25.90 ± 0.49	1.9	3.6
50	49.50 ± 2.33	4.7	1.0
Mean		5.12	
Inter-assay (reproducibility)			
2	2.00 ± 0.18	9.2	0
5	5.30 ± 0.49	9.3	6.0
10	10.40 ± 0.59	5.7	4.0
25	24.30 ± 0.69	2.8	2.8
50	50.20 ± 0.37	0.7	0.4
Mean		5.5	

fluid. The detection limit was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1.

Precision

The data presented in Table I demonstrate the within-batch (intra-assay) and between-batch (inter-assay) variation of the method. Intra-assay variability was determined at five concentrations in quadruplicate at levels of 2–50 ng/ml of the drug in plasma. Inter-assay variability was determined singly at the same five concentrations in four replicate runs. The precision of the method (mean coefficient of variation, C.V.) for the values of the recovered determinate standards when calculated as unknown against the generated linear regression line were 5.1 and 5.5% for intra- and inter-assay, respectively.

The precision study was not carried out for milk samples as the precision of the assay for milk was found to be very similar to that of plasma.

Linearity

Measures of linearity as defined by the correlation coefficients (r) of the regression lines generated based on duplicate calibration curves obtained for plasma and milk were consistently better than 0.999.

Recovery

In order to establish the recovery, two calibration curves based on the external standard method were set up. One calibration curve was based on spiked plasma samples in the concentration range 2–50 ng/ml and the other was based on working reference standards in the same concentration range. The overall recovery was calculated by comparing the slopes of the regression lines generated for the

TABLE II

RECOVERY FROM PLASMA AND FROM MILK

Concentration (ng/ml)	Tripelennamine peak heights (mean of duplicates) (cm)		Recovery (%)
	Set A: working standards	Set B: standards extracted from plasma/milk	
Plasma			
2*	0.9	0.7	78
5	0.9	0.7	78
10	1.9	1.5	79
25	4.5	3.5	79
50	9.1	7.1	77

Mean overall recovery = 78%
Regression line for set A: $y = 0.18133x + 0.0046$, $r = 0.9999$
Regression line for set B: $y = 0.14036x + 0.02612$, $r = 0.9999$

$$\text{Overall recovery} = \frac{0.14036}{0.18133} \times 100 = 77\%$$

Mean recovery determined by the two methods: 77.5%

Milk			
5	0.50	0.35	70
10	0.85	0.65	77
25	2.30	1.75	76
75	7.90	5.70	72
150	15.75	11.90	76
300	31.00	22.60	73

Mean overall recovery = 74%
Regression line for set A: $y = 0.10395x - 0.06310$, $r = 0.999$
Regression line for set B: $y = 0.07612x - 0.00869$, $r = 0.999$

$$\text{Overall recovery} = \frac{0.07612}{0.10395} \times 100 = 73\%$$

Mean recovery determined by the two methods: 73.5%

*More sensitive detector setting.

two sets. The same precedure was applied to the milk samples in the concentration range 5–300 ng/ml tripelennamine. The overall recovery was also calculated by direct comparison of the peak heights. The results obtained are shown in Table II.

Applications of the method

The described method has been successfully applied to the samples from cattle receiving an intravenous injection of a formula containing tripelennamine. For reasons of confidentiality, we were not given permission to reveal the results of the analysis of plasma and milk samples. However, chromatograms from the actual plasma and milk samples are included in Fig. 2. As can be seen, these chromatograms resemble the chromatograms obtained from spiked samples and no interferences were observed from endogenous components of plasma or milk.

CONCLUSION

For routine analysis of tripelennamine in bovine plasma and milk, a new method of analysis based on direct injection/column-switching technique was developed. Plasma samples could be injected directly while milk samples had to be centrifuged first, to separate fat, prior to injection. The method is rapid and eliminates the need for lengthy and tedious liquid–liquid extraction procedures with good recovery and low running cost. The method achieves a low limit of detection comparable with some reported GC methods which require long and elaborate extraction procedures. The chromatography was selective, i.e. there were no interferences from endogenous plasma components, and sharp peaks were obtained for the drug and the internal standard.

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