Journal of Chromatography, 342 (1985) 315—321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2643

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CERTAIN SALICYLATES AND THEIR MAJOR METABOLITES IN PLASMA FOLLOWING TOPICAL ADMINISTRATION OF A LINIMENT TO HEALTHY SUBJECTS

D. DADGAR*,*, J. CLIMAX, R. LAMBE and A. DARRAGH

Institute of Clinical Pharmacology, P.O. Box 469, 1 James's Street, Dublin 8 (Ireland)

(First received November 9th, 1984; revised manuscript received March 22nd, 1985)

SUMMARY

The liniment used is a topical analgesic and anti-inflammatory preparation containing two active constituents, 3-phenylpropylsalicylate and ethyl-5-methoxysalicylate, in solution in isobutyl decanoate. It is known that 3-phenylpropylsalicylate is metabolised to salicylic acid and salicyluric acid and ethyl-5-methoxysalicylate is metabolised to 5-methoxysalicylic acid and gentisic acid. In the present study the separation of the salicylates and their metabolites was carried out on a Waters μBondapak C_{18} column using two different mobile phases, methanol—water (80·20) for the parent drugs and methanol—5% aqueous acetic acid (27:73) for their metabolites. The salicylates and their metabolites were detected by absorption at 310 nm. The limits of detection for parent drugs and metabolites were respectively 0.2 and 0.1 μg/ml in plasma, using a 1-ml plasma sample and a 20-μl injection from a reconstituted volume of 250 μl. Mean percentage coefficients of variation for intra-assay and inter-assay precision were between 3.3 ± 1.9% to 9.1 ± 3.7% and 6.8 ± 2.2% to 15.7 ± 10.1%, respectively. Linearity, as measured by the correlation coefficient of intra-assay linear regression curves, was better than 0.998 in all cases.

INTRODUCTION

The liniment is composed of two active constituents, 3-phenylpropylsalicylate (PPS) and ethyl-5-methoxysalicylate; (EMS) in solution in isobutyl decanoate. Both PPS and EMS have anti-inflammatory and analgesic properties. The penetration of EMS through skin is rapid, while that of PPS is slower. The

*Present address School of Chemical Sciences, National Institute for Higher Education, Dublin 9, Ireland.

0378-4347/85/$03.30

combination of EMS and PPS as a solution in isobutyl decanoate produces a synergistic analgesic and anti-inflammatory action.

The present study was undertaken to evaluate the percutaneous absorption of the liniment by determining PPS, EMS and its metabolites gentisic acid (GA), salicyluric acid (SU), 5-methoxysalicylic acid (MSA) and salicylic acid (SA) in blood plasma. Although a number of methods are available for quantifying salicylates in biological fluids [1—18], most of these methods lack sufficient sensitivity and only refer to assay of SA, SU and GA. In this paper a simple high-performance liquid chromatographic (HPLC) procedure for measuring PPS, EMS and their metabolites in plasma is described. The method was used to determine percutaneous absorption of the active constituents when 10 g of the linement were topically applied to the skin of healthy volunteers.

EXPERIMENTAL

Reagents

EMS, PPS and MSA were obtained from P.C.A.S. (France), SA from Baker, SU and GA from Aldrich, methanol and ethyl acetate (HPLC grade) from Fisons, and acetic acid (Aristar grade) and hydrochloric acid (1 *M*, analytical-reagent grade) from BDH (U.K.).

For the preparation of plasma standards, dried human plasma (from the Blood Transfusion Service Board, Ireland) was dissolved in nanopure water (deionised water obtained by reverse osmosis, Barnstead system). The blank plasma obtained was examined for the presence of endogenous components which might interfere with the salicylates and their metabolites in the assay system. The reconstituted plasma was stored at 4° C and used within two weeks of preparation.

Instrumentation

The HPLC system consisted of a Varian 5000 liquid chromatograph solvent delivery system equipped with a Waters U6K manual injector and fitted with a Waters Assoc. μBondapak C_{18} reversed-phase column (30 × 0.39 mm I.D., particle size 10 μm). A Pye-Unicam LC-3 variable-wavelength UV detector was used. Chromatograms were recorded on a Philips Model PM 8251 single-pen recorder.

Chromatography

The chromatographic conditions for separation of parent drugs were set as follows: mobile phase, methanol—water (80:20); flow-rate, 1.0 ml/min; recorder chart speed, 0.5 cm/min; detection wavelength, 310 nm; injection volume, 20 μl. Under the described chromatographic conditions the mean retention times for EMS and PPS were 3.6 min and 7.0 min respectively (Fig. 1A).

The chromatographic conditions for the separation of the metabolites were set as follows: mobile phase, methanol—5% aqueous acetic acid (27:73); flow-rate, 1.5 ml/min; recorder chart speed, detection wavelength and injection volume were the same as those set for the parent drugs. Under the described chromatographic conditions the mean retention times were 4.0 min for GA, 5.0 min for SU, 10.0 min for SA and 11.4 min for MSA (Fig. 1B).

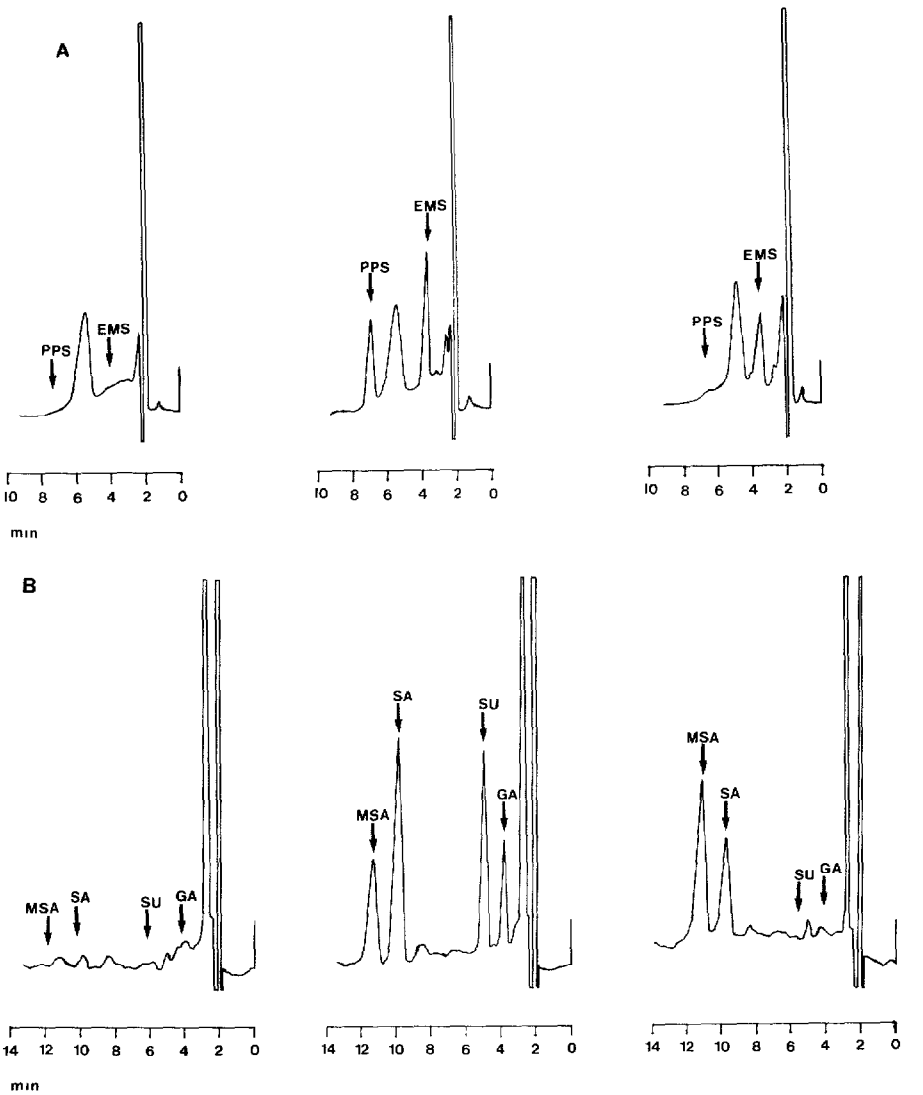

Fig. 1 (A) Chromatograms of extracts from the plasma of one of the volunteers (subject B.W.). Left: Pre-topical dose; middle: drug-free plasma spiked with 1 μg/ml EMS and PPS; right. 6 h post topical dose. (B) Chromatograms of extracts from the plasma of one of the volunteers (subject B.W.). Left· Pre-topical dose; middle. drug-free plasma spiked with 0.5 μg/ml of each of the metabolites; right: 6 h post topical dose.

Standards

From each of the salicylates and their metabolites 10.00 mg were accurately weighed and dissolved in 100 ml methanol to yield the stock standard solution (100 μg/ml). This stock solution was further diluted to give working standards ranging from 1 to 100 μg/ml. Spiked plasma standards were prepared each day by addition of 100 μl of the working standard solutions to 1 ml plasma, to provide standards ranging from 0.1 to 10 μg/ml of the drugs in plasma.

TABLE I

INTRA-ASSAY VARIATION FOR THE SALICYLATES AND THEIR METABOLITES

$\bar{x}$ = Mean concentration found, S D = standard deviation (n = 4), C.V. = coefficient of variation (%), r = correlation coefficient

Concentration added (μg/ml)	Concentration found (μg/ml)											
	PPS		EMS		SA		MSA		SU		GA	
	$\bar{x}$ ± S.D	C V	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S.D	C V.	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S D	C V
0 50	0 59 ± 0 04	6 8	0 51 ± 0 07	13 7	0 60 ± 0.04	6 6	0 66 ± 0 13	19 6	0.54 ± 0.05	9 3	0 59 ± 0 02	3.4
1 00	0 94 ± 0 04	4 2	0 96 ± 0 13	13 5	0 98 ± 0 11	11 2	0 93 ± 0 04	4 3	0 98 ± 0 06	6.1	1 01 ± 0 09	8 9
2 00	2.02 ± 0.07	3 5	2 07 ± 0 23	11 1	1 96 ± 0 08	4 1	1 89 ± 0.09	4.8	2 05 ± 0 05	2 4	2 00 ± 0 06	3 0
4 00	3 94 ± 0.04	1.0	3 81 ± 0 25	6 5	3 92 ± 0 11	2 8	4 01 ± 0 09	2 2	3 88 ± 0 11	2 8	3 86 ± 0.18	4 7
6 00	6 04 ± 0 09	1 5	6 06 ± 0 49	8.1	6.19 ± 0 25	4 0	6 14 ± 0 17	2 7	6.16 ± 0 13	2 1	6 12 ± 0 09	1.5
8 00	7 94 ± 0 26	3 3	8 34 ± 0 34	4 1	7 66 ± 0 39	5 1	7 69 ± 0 35	4 4	7 79 ± 0 12	1.5	7 74 ± 0 18	2 3
10 00	10 06 ± 0 28	2 8	9 76 ± 0 64	6 5	10 19 ± 0 26	2 5	10 18 ± 0 49	4 8	10 11 ± 0 19	1 9	10 19 ± 0 23	2 3
Mean C V ± S D	3 3 ± 1 9		9 1 ± 3 7		5 2 ± 3.0		6 1 ± 6 0		3 7 ± 2 9		3 7 ± 2 5	
r	0 9999		0 9986		0 9987		0 9988		0 9994		0 9991	

TABLE II

INTER-ASSAY VARIATION FOR THE SALICYLATES AND THEIR METABOLITES

$\bar{x}$, S D and C V as in Table I

Concentration added (μg/ml)	Concentration found (μg/ml)											
	PPS		EMS		SA		MSA		SU		GA	
	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S.D.	C V	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S D	C V	$\bar{x}$ ± S D	C V
0 50	0 53 ± 0 18	33 9	0 51 ± 0 11	21 5	0 68 ± 0 06	8 8	0.55 ± 0 16	29.1	0 51 ± 0 08	15 7	0 49 ± 0 07	14 3
1.00	0 93 ± 0 22	23.6	0 99 ± 0 03	3 0	1 05 ± 0 07	6 7	0 98 ± 0 09	9.2	0 84 ± 0.06	7 1	0 90 ± 0 18	20 0
2 00	1 97 ± 0 23	11 6	2 05 ± 0 08	3 9	2 14 ± 0 13	6 1	1 95 ± 0 17	8 7	1 96 ± 0 10	5.1	1.99 ± 0 18	9 0
4 00	3 86 ± 0 52	13 5	3 95 ± 0 39	9 9	4 14 ± 0 11	2 6	3 96 ± 0 07	1 8	4 15 ± 0 13	3 1	4 10 ± 0 32	7 8
6 00	6 28 ± 0 72	11 5	6 02 ± 0 28	4 6	6 21 ± 0 41	6 6	6 18 ± 0 50	8 1	6 22 ± 0 44	7.1	6 13 ± 0 70	11 4
8 00	7 84 ± 0 19	2 4	7 87 ± 0 51	6 5	7 93 ± 0 64	8 1	7 84 ± 0 70	8 9	8 01 ± 0 58	7 2	8 02 ± 0 77	9.6
10 00	9 96 ± 1 35	13 5	10 10 ± 0 47	4 6	9 86 ± 0 88	8 9	10 06 ± 1 18	11.7	9.83 ± 0.80	8.1	9 88 ± 1 10	11 1
Mean C V ± S D	15 7 ± 10 1		7 7 ± 6 5		6 8 ± 2.2		11 1 ± 8 5		7 6 ± 3 9		11 9 ± 4.1	

Extraction procedure

Parent drugs (EMS and PPS). Plasma (1 ml) spiked with 100 μl working standards was mixed with 1 ml nanopure water in a glass-stoppered tube. After the addition of 7 ml ethyl acetate, using an all-glass dispenser, the drugs were extracted by rotating the tubes gently for 15 min on a mechanical rotator, followed by centrifugation for 15 min at 700 *g* at 0°C. Exactly 5 ml of the supernatant were transferred into a glass tube which was placed in a water bath at 40°C and the solvent was evaporated under a gentle stream of oxygen-free nitrogen. The residue was reconstituted in 250 μl methanol and 20 μl were injected for HPLC analysis.

Metabolites. The extraction procedure for the metabolites was exactly the same as that for the parent drugs except that a 2-ml aliquot of 0.05 *M* hydrochloric acid was added in place of nanopure water, before ethyl acetate extraction. Samples were extracted exactly as described above for standards except in place of the working standards an equivalent amount of methanol was added.

Calibration and calculation

Each calibration curve was obtained by linear regression of the peak heights of each compound versus concentrations of that compound (external standard method). These calibration curves were then used to interpolate the concentration of salicylates and their metabolites in plasma from the measured peak height of each individual compound.

RESULTS AND DISCUSSION

Limit of detection

Under procedural conditions the limits of detection, using a 1-ml plasma sample and 20-μl injections, were 0.2 and 0.1 μg/ml of the salicylates and their metabolites, respectively. The limit of detection was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1. Higher sensitivity can be obtained by dissolving the extract residue in the mobile phase rather than in methanol. This will allow the injection of a larger sample volume. However, dissolution of extract residue in the mobile phase results in a cloudy solution which will have to be filtered prior to injection.

Reproducibility

The data presented in Tables I and II demonstrate the within-batch (intra-assay) and between-batch (inter-assay) variation of the method. Intra-assay variability was determined at seven concentrations in quadruplicate: 0.50, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 μg/ml of each compound in plasma. Inter-assay variability was determined singly at the same seven concentrations in four replicate runs. The precision of the method (mean coefficient of variation ± standard deviation) for the values of recovered determinate standards, when calculated as "unknown" against the linear regression line were between 3.3 ± 1.9% to 9.1 ± 3.7% and 6.8 ± 2.2% to 15.7 ± 10.1% for intra-assay and inter-assay variation, respectively.

TABLE III

PLASMA LEVEL OF SALICYLATES AND THEIR MAJOR METABOLITES IN ONE OF THE VOLUNTEERS (SUBJECT B.W.)

N.D. = Not detectable.

Time	Concentration (μg/ml)					
	EMS	PPS	GA	SU	MSA	SA
Pre-Dose	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2 h	0.5	N.D.	N.D.	N.D.	0.7	0.1
6 h	0.6	N.D.	N.D.	N.D.	0.7	0.3
12 h	0.3	N.D.	N.D.	N.D.	0.5	0.3
24 h	0.2	N.D.	N.D.	N.D.	0.7	0.2

Linearity

A measure of linearity, as defined by the correlation coefficient of the regression lines for each compound, is given under intra-assay variation (Table I). The correlation coefficients were better than 0.998 in all cases.

Recovery

Recovery was calculated by comparing the peak heights of parent drugs and metabolites after their extraction from plasma with the peak heights of series of unextracted reference standards. In the concentration range 0.5—10 μg/ml, the mean overall percentage recoveries were 81.2 ± 6.7% for EMS, 84.5 ± 8.2% for PPS, 62.1 ± 3.8% for GA, 66.7 ± 8.2% for SU, 73.4 ± 9.1% for SA and 67.3 ± 10.2% for MSA.

Plasma levels of salicylates and their metabolites

The purpose of the present study was to determine the disposition of the salicylates and their major metabolites following topical administration of the liniment to healthy volunteers. All subjects gave written informed consent to participate in the study, the protocol for which was approved by the Institutional Review Board. The liniment (10 g) was applied to the skin of the chest and back of the subjects. Blood (10 ml) was collected from an antecubital vein into pre-cooled (4°C) fluoride—oxalate vacutainers at the following times: pre-application and 1, 3, 6, 12 and 24 h after application. The plasma was separated within 15 min and stored at —20°C before analysis. Table III presents typical results from one of the volunteers using the described procedure. These data confirmed that percutaneous absorption of the active constituents of the liniment had occurred.

REFERENCES

1 I. Bekersky, H.G. Boxenbaum, M.H Whitson, C.V. Puglisi, R. Pocelinko and S.A. Kaplan, Anal. Lett., 10 (1977) 539.
2 G.W. Peng, M.A.F. Gadalla, V. Smith, A. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 710.

3 D. Blair, B.H. Rumack and R.G. Peterson, Clin. Chem., 24 (1978) 1543.
4 E.N. Amick and W.D. Mason, Anal. Lett., 12 (1979) 629.
5 M. Mandelli and G. Tognoni, Clin. Pharmacokin., 5 (1980) 424.
6 B.E. Cham, F. Bochner, D.M. Imhoff, D. Johns and M. Rowland, Clin. Chem., 26 (1980) 111.
7 L. Yee Lo and A. Bye, J. Chromatogr., 181 (1980) 473.
8 C.P. Terwij-Groen, S. Heemstra and J.C. Kraak, J. Chromatogr., 181 (1980) 385.
9 D.L. Maulding and J.F. Young, J. Pharm. Sci., 69 (1980) 1224.
10 R.O. Day, S.H. Dromgoole, D.E. Furst, C. Hignite and H.E. Paulus, J. Pharm. Sci , 70 (1981) 1090.
11 S.M. Douidar and A.E. Ahmed, J. Clin. Chem. Clin. Biochem., 20 (1982) 791.
12 U. Reidl, J. Chromatogr., 272 (1983) 325.
13 K. Selinger and W.C. Purdy, Anal. Chim. Acta, 149 (1983) 343.
14 S.T. Ingalls, J.F. Schwerkoske, W.B. Forman and C.L. Hoppel, J. Chromatogr., 274 (1983) 366.
15 S.K. Bakar and S. Niazi, J. Pharm. Sci., 72 (1983) 1020.
16 W.D. Mason and R.B. Gillilan, Anal. Lett., 16 (1983) 903.
17 M. Nieder and H. Jaeger, J. High Resolut Chromatogr. Chromatogr. Commun., 6 (1983) 655.
18 J.N. Buskin, R.A. Upton and R.L. Williams, Clin. Chem., 28 (1982) 1200.