Journal of Chromatography, 231 (1982) 492—496
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1334

Note

High-performance liquid chromatographic procedure for simultaneous determination of SKF 78729A and its N-acetyl metabolite

R.N. GUPTA*

Department of Pathology, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

M.R. ACHONG

Department of Medicine, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

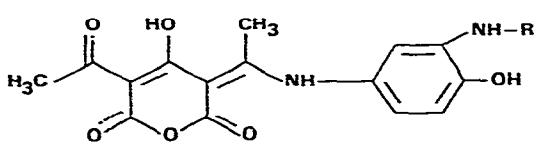
and

F. ENG

Department of Pathology, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

(First received February 2nd, 1982; revised manuscript received March 29th, 1982)

SKF 78729A {5-acetyl-4-hydroxy-3-[1-[(3-amino-4-hydroxyphenyl)amino] ethylidene] 2H-pyran-2,6(3H)-dione hydrochloride} is a compound which can inhibit immediate-type allergic reactions in vitro and in animal models [1]. It is currently being tested in humans as an inhibitor of allergen-provoked bronchospasm. This paper describes a high-performance liquid chromatographic (HPLC) procedure for the simultaneous estimation of SKF 78729A and its N-acetyl metabolite (Fig. 1) in human plasma.



1. R=H
2. R=$COCH_3$
3. R=COC_2H_5

Fig. 1. Structural formulae: 1 = SKF 78729A; 2 = N-acetyl derivative of SKF 78729A; 3 = N-propionyl derivative of SKF 78729A.

0378-4347/82/0000—0000/$02.75

MATERIALS AND METHODS

Reagents were of analytical grade and the solvents were distilled in glass by the supplier (Caledon Labs., Georgetown, Canada). Solutions of 1 mg/ml of each of SKF 78729A (1) and its N-acetyl metabolite (2) were prepared in 1% acetic acid in methanol. A solution of 0.1 mg/ml of the N-propionyl derivative of SKF 78729A (3) was prepared in 1% acetic acid in methanol. Compounds 1, 2 and 3 were obtained from Smith Kline and French Canada, Mississauga, Canada. These solutions were stored in dark bottles at 4°C. Plasma standards, 4 μg/ml, of 1 and 2 were prepared from drug-free plasma and appropriate volumes of solutions of 1 and 2. Plasma standards of 2, 1, 0.5 and 0.25 μg/ml, of compounds 1 and 2, were prepared by serial dilution. Plasma standards were divided into 1-ml aliquots and stored frozen. Working internal standard solution (2 μg/ml) was prepared when required by diluting stock solution of 3 1:50 with 1% aqueous acetic acid. After an oral dose of 500 mg of SKF 78729A had been given to healthy volunteers, blood was collected in heparinized evacuated blood collecting tubes. Plasma was collected in plastic test tubes and kept frozen until analyzed.

Sample preparation

Bond Elut® C_{18} disposable extraction columns of 1-ml capacity and a Vac Elut® system were purchased from Analytichem International (Harbor City, CA, U.S.A.). The columns were washed under suction once with 2% acetic acid, twice with methanol and finally with 2% acetic acid. Plasma (0.5 ml) and working internal standard solution (0.5 ml) were applied to the washed columns placed in the suction rack with the suction turned off. Pressure was applied to the columns with a stream of nitrogen so that the liquid passed through the column in 40—60 sec. The columns were washed twice with 0.2% acetic acid and the washings discarded. The columns were then eluted with 0.5 ml of methanol containing 2 μl per 100 ml mercaptoethanol. The eluate was collected in 75 × 12 mm disposable glass tubes and evaporated at room temperature with a current of nitrogen. The residue in each tube was dissolved in 50 μl of mobile phase and 20 μl were injected into the liquid chromatograph.

Chromatography

The assay was performed with an HPLC system consisting of Model 100 A pump, and an Hitachi Model 100-40 variable-wavelength absorption detector set at 330 nm (Altex, Berkeley, CA, U.S.A.). Injections were made by means of a Rheodyne Model 7125 syringe loading injector with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). The chromatogram was recorded and the peaks were integrated with a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

A 10-μm PRP-1 (XAD resin), 15 cm × 4.1 mm I.D. (Hamilton, Reno, NV, U.S.A.) column was used. The mobile phase was prepared by mixing acetonitrile (700 ml) and glass-distilled water (1 l) containing 70% perchloric acid (0.5 ml) and 24% methanolic solution of tetramethylammonium hydroxide (0.5 ml). The pH of the mobile phase was 3.0. The flow-rate was 0.8 ml/min with a back pressure of 5.54 MPa (800 p.s.i.).

RESULTS AND DISCUSSION

SKF 78729A and its N-acetyl metabolite cannot be extracted efficiently into water-immiscible solvents such as chloroform or ethyl acetate. These compounds are isolated in yields of 70—90% by the procedure described in this report. The ratios of drug/internal standard and/or metabolite/internal standard remain constant in this recovery range. The recovery of drug is reduced drastically if the mixture of plasma and internal standard passes through the column in less than 30 sec. Extraction with acetonitrile and separation of organic layer by freezing the aqueous layer is less efficient than extraction by Bond-Elut columns.

Fig. 2B shows a chromatogram of the extract of drug-free plasma. There are virtually no peaks due to endogenous components of plasma. For convenience, blood is withdrawn from patients in commercially available evacuated blood-collecting tubes. The undeclared additives used in the manufacturing of those tubes affect trace drug analysis either by causing interference or by affecting the distribution of drug in cells and plasma water. It has been claimed that heparinized Venoject® brand tubes are suitable for drug analysis [2]. However, blood collected in these tubes produces additional peaks when analyzed for SKF 78729A by the described procedure. Blood collected in newly formulated blue-capped heparinized Vacutainer® brand tubes (Becton Dickinson, Orangeburg, NY, U.S.A.) does not produce any peak when analyzed for compound 1 by the present procedure. It has recently been claimed that drug concentrations are not significantly affected when blood is collected in these tubes [3].

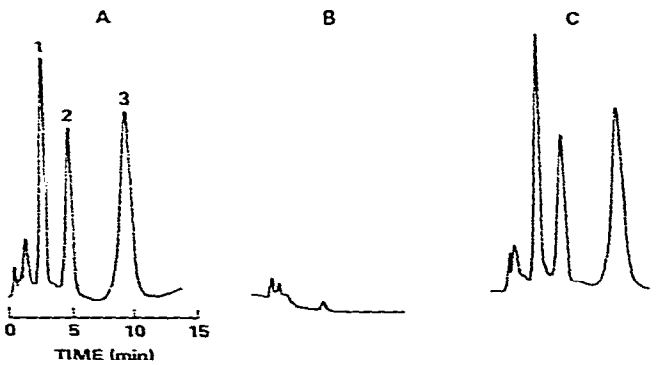


Fig. 2. Chromatograms (detector = 0.05 A full scale; plotter attenuation = 2; 4 mV) of: (A) a mixture of methanolic solutions of compounds 1, 2 and 3, (b) an extract of drug-free plasma, (c) an extract with added 1 and 2 (1 μg/ml). Peaks: 1 = SKF 78729A; 2 = metabolite; 3 = internal standard.

N-Acetylation of primary aromatic amino groups is a major inactivation step for many drugs [4]. However, formation of the N-acetyl derivative of SKF 78729A has not yet been unequivocally established in man. We have analyzed the extracts of plasma obtained from volunteers who had ingested oral doses of compound 1 with different columns (Ultrasphere-ODS, 5 μm, 25 cm × 4.6 mm I.D., and Ultracil-Octyl, 10 μm, 25 cm × 4.6 mm I.D.),

and different composition of mobile phase (acetonitrile—methanol—water and 2-ethylhexylphosphoric acid). In every case the retention time of the additional peak present in the volunteers specimen was identical with that of the N-acetyl derivative of the drug.

Fig. 2C shows a chromatogram of an extract of plasma standard. The peaks of drug, metabolite and internal standard are sharp and are well separated. The standard curve is linear for the range tested (0.25—4 μg/ml) for both the drug and its N-acetyl metabolite either by comparing the ratios of peak heights or by comparing the ratios of peak areas of the drug or of the metabolite and the internal standard. The calibration curve passes through the origin. Analysis of plasma supplemented with the drug and its metabolite showed a within-batch coefficient of variation (C.V.) of 6.5% for the drug (n = 10, mean = 1.8 μg/ml) and 7.6% for the metabolite (n = 10, mean = 1.8 μg/ml). Analysis of plasma over a period of ten weeks showed a between-batch C.V. of 7.5% for the drug (n = 20, mean = 1.9 μg/ml) and 4.0% for the metabolite (n = 20, mean = 1.9 μg/ml).

This procedure has been used to study the bioavailability of SKF 78729A in humans. An example of a plasma concentration—time curve for the drug and its metabolite in a volunteer given 500 mg of SKF 78729A orally is shown in Fig. 3. The procedure allows detection of 200 ng/ml of drug or of metabolite. For the detection of as low as 25 ng/ml of drug or of metabolite 1 ml of the specimen is used and the residue of plasma extract is dissolved in 25 μl of mobile phase.

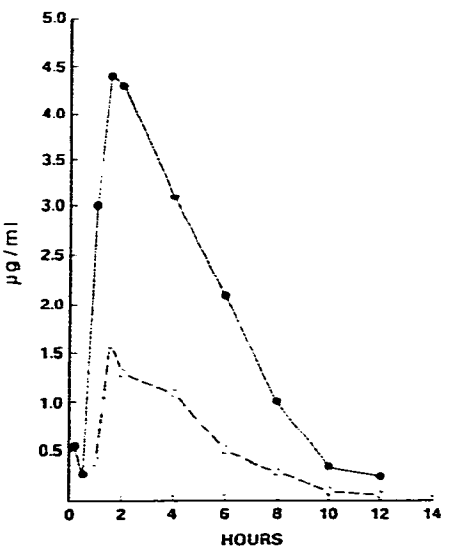


Fig. 3. Plasma drug concentration—time curve in a healthy volunteer after ingestion of 500 mg of SKF 78729A. (•), SKF 78729A; (○), N-acetyl derivative.

ACKNOWLEDGEMENT

This work was supported by a grant from Smith Kline and French, Canada Ltd.

REFERENCES

1 L.W. Chakrin and R.D. Krell, J. Pharmacol. Exp. Ther., 207 (1978) 756.
2 R.C. Veith and C. Perera, N. Engl. J. Med., 300 (1979) 504.
3 B. Vinet, Clin. Biochem., 14 (1981) 105.
4 S.C. Glauser, Med. Clin. North Amer., 58 (1974) 945.