The acetylated compound 1c shows no anticonvulsant activity at all. This result also corresponds with the proposed pharmacophore although a direct comparison of compound 1c with 1b is limited because of the difference in the experimental partition coefficient octanol/water (log P of 1b = 1.39; log P of 1c = 0.08).
A correct distance range between the exocyclic NH_2-group and the endocyclic NH-group and also the free access to these functionalities are essential for anticonvulsant activity. Deviations of this core structure this can also mean more polar groups in the heterocyclic system result in inactive compounds like 2a. Therefore all 5-amino-pyrazole-3-one 2, 3 show no anticonvulsant activity.

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Dr. Hans-Joachim Lankau
Arzneimittelwerk Dresden GmbH
Meißner Straße 35
D-01445 Radebeul

Research Institute for Industrial Pharmacy and Department of Pharmaceutical Chemistry, Potchefstroom University for Christian Higher Education, Potchefstroom, South Africa

Reverse-phase liquid chromatographic UV method for analysis of hexoprenaline sulphate and selection of chromatographic conditions suitable for multi-β-agonist analysis

M. M. DE VILLIERS and J. J. BERGH

Hexoprenaline is a widely prescribed potent β_2-agonist [1]. It appears to be mostly used as a stimulant in obstetric practice [2]. Besides this use as a tocolytic it also relieves reversible airway obstruction, including status asthmaticus [1]. Although, this drug is widely used, drug standards for hexoprenaline have not been published in major compendia. This has necessitated the need to develop specific assay procedures for hexoprenaline in the presence of related compounds and potential degradation products.
A number of single and multi-component high performance liquid chromatographic (HPLC) methods have been reported for the analysis of β_2-agonists similar in structure to hexoprenaline [3–5]. The application of HPLC is limited when determining β_2-agonists in bio-samples because of insufficient selectivity and lack of sensitivity of common HPLC detectors [4]. Consequently HPLC is rarely used to determine the illicit use of the compounds in zootechnics and in sports [4]. GC separations of these compounds after purification of analytes by means of either liquid-liquid partition or solid-phase extraction are used to overcome these problems [6]. However, HPLC has significant advantages over GC for the analysis of these compounds in pharmaceutical dosage forms [4, 5, 7]. Unfortunately no reported method specific for hexoprenaline, either as the sulphate or hydrochloride salts, could be found. In this study the applicability of HPLC for assaying hexoprenaline sulphate in pharmaceutical dosage forms was investigated.
Isocratic elution (Fig. 1) was performed using a mobile phase of methanol and water (45 : 55 v/v) containing 0.1 M ammonium acetate and 0.1 M triethylamine with 0.1 M formic acid at a pH of 3.5 [4]. Fig. 1 shows the chromatogram obtained for the direct injection of a sample containing hexoprenaline · H_2SO_4 75 μg/ml and spiked with clenbuterol 50 μg/ml. The capacity factor, k′, for hexoprenaline was 2.96 and for clenbuterol 1.20 showing satisfac-

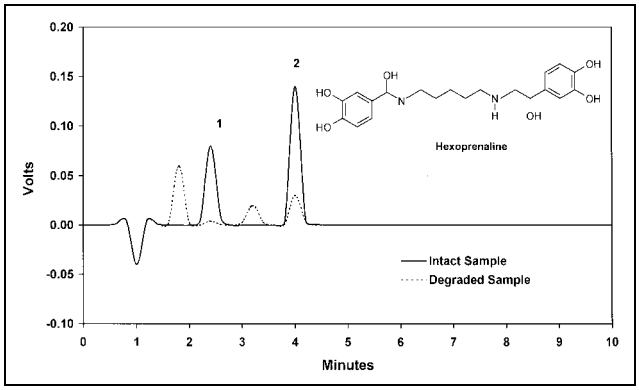

Fig. 1: HPLC chromatogram (285 nm) produced after injection 50 μl of a sample containing 75 μg/ml hexoprenaline sulphate (2) and spiked with 50 μg/ml clenbuterol (1). Mobile phase 45% methanol in buffer with pH about 3.8

tory resolution. The column dead time (t_0) where $k' = 0$ was 1.09 min. An example of a chromatogram of a sample degraded by boiling it in 1 M HCl for 1 h under reflux is given as the broken line in Fig. 1. Degradation products did not interfere with analyte peaks. The method showed good repeatability (RSD = 0.76%, n = 10) and the system appeared to be robust over a period of two weeks in which more than 200 samples were processed.

Sometimes a small shift in retention time of hexoprenaline (4–12 s) was observed. The linearity of the isocratic system was demonstrated by constructing calibration curves in the range 50–150 µg/ml. Least squares analysis resulted in coefficients of variation close to unity for (mean = 0.9996, RSD = 0.0011, n = 12) that confirm the linear working concentration in the assay. This data was obtained by replicate analysis of standard solutions at six concentrations within the linearity range. Assay recoveries for hexoprenaline, determined by spiking commercial samples, and precision for 5 determinations from the tablets, inhalation solution and syrup were 99.06 ± 1.36%.

Satisfactory isocratic separation of all the β-agonists chosen was impossible and thus linear gradient elution was explored [4]. For linear gradient binary elution (Fig. 2) water was used as solvent A and a mixture of methanol and water (95 : 5 v/v) as solvent B. A buffer consisting of 0.1 M ammonium acetate and 0.1 M triethylamine, adjusted to pH 3.5 with 0.1 M formic acid, was added to solvent A and B [4]. This was done to avoid ions dilution in the mobile phase under gradient elution owing to the increment of the percentage of organic solvent, and thus to prevent possible disturbances on ionisation processes [8]. The result of a suitable linear gradient elution obtained with trial and error optimisation is shown in Fig. 2, and optimal gradient conditions are depicted in the insert. Two important aspects had to be considered during linear elution. First, to obtain sufficient retention of the first eluting analytes (isoprenaline and salbutamol) the initial mobile phase has to be of low elutropic strength (methanol <5%, up to 5 min). Secondly the application of a rather steep gradient (from 5 to 60% methanol after 5 min until 10 min) was necessary to obtain sharp peaks (sensitivity) for the other compounds.

The robustness of the linear gradient procedure was tested by the injection of more than 40 samples containing various concentrations of the drugs, checking the performance. For example the mean area under the peak for hexoprenaline shown in Fig. 2 was 242365, RSD = 0.48% (n = 9).

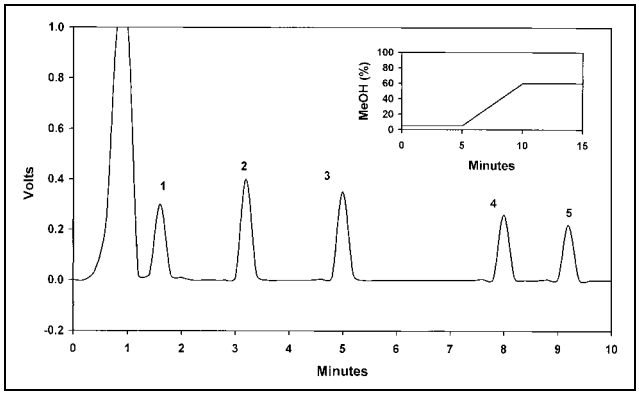

Fig. 2: HPLC chromatogram (285 nm) of the optimised separation obtained for a mixture of β-agonists with linear gradient elution conditions as shown in the insert. Sample injection volume was 50 µl containing 75 µg/ml of each analyte. Peaks: 1: isoprenaline; 2: salbutamol; 3: fenoterol; 4: clenbuterol; 5: hexoprenaline

It appeared that both retention and peak shape of the analytes stayed constant. The mean retention time for hexoprenaline injected in different concentrations and over several days was 9.2 min with a deviation of ±12 s (n = 40). The applicability of the gradient conditions for urine analysis using HPLC in combination with MS detection might be a matter of future research.

This accurate and precise reverse phase HPLC method was specific for the determination of hexoprenaline in the presence of its related compounds and potential degradation products in raw materials and pharmaceutical dosage forms. The viability, reproducibility, and accuracy of the proposed procedure in the quantitative analysis of hexoprenaline in tablets, inhalation solutions and syrup dosage was also demonstrated.

Experimental

1. Equipment

The following HPLC system was used: two pumps (LC-6A); system controller (SCL-6B); auto injector system (SIL-6B); integrator system (C-R4A); UV-VIS detector (SPD-6AV) all from Shimadzu Corporation (Japan). All flow rates were set at 1 ml/min and UV absorbance was measured at 285 nm [3, 4]. Analyses were performed using a Nova Pak C_{18}, 4 µm 10 cm × 8 mm i.d. Radial Pak liquid chromatographic cartridge (Waters, Milford, MA, USA) inside a Waters RCM 8 × 10 compression module. All reagents were HPLC or analytical grade, and were used without further purification. HPLC grade water was prepared by purifying demineralised warer in a Milli-Q system (Millipore, Bedford, MA, USA).

2. Substances

The β-agonists, isoprenaline · H_2SO_4, salbutamol · H_2SO_4, fenoterol · HCl, clenbuterol · HCl and hexoprenaline · H_2SO_4, all with purity >99%, were obtained from Sigma Chemical Corporation (St. Louis, MO, USA) or from Adcock Ingram (Industria, Johannesburg, South Africa). All hexoprenaline pharmaceuticals were Ipradol® products from Continental Ethicals (Johannesburg, South Africa).

3. Procedure

Separate stock solutions of the β-agonists (1 mg free base/ml) were prepared in HPLC water containing 5% of methanol. The only exception was isoprenaline which was dissolved in pure methanol. For analysis of standard solutions suitable dilutions were made in HPLC water. An inhalation solution (0.25 mg/ml), syrup (0.125 mg/5 ml) and tablets (0.5 mg) were tested. Tablets were mixed with 10 ml water and, after ultrasonication for 15 min, the sample solution was filtered. The filtered solution, syrup or inhalation solution was suitably diluted with water to the desired concentration prior to analysis.

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Melgardt M. de Villiers, Ph. D.
Research Institute for Industrial Pharmacy
Potchefstroom University for
Christian Higher Education
Potchefstroom, 2520
South Africa