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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIFFERENTIAL PULSE POLAROGRAPHIC DETECTION FOR ASSAYING DRUGS IN FEED

STABILITY-INDICATING ASSAY OF DIACETOLOL

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SUMMARY

Reversed-phase high-performance liquid chromatography with differential pulse polarographic detection at a dropping mercury electrode provides a high degree of specificity for stability-indicating assays of drugs in animal feed. The selectivity of this detection system is demonstrated in the assay of diacetolol in feed at concentrations down to 50 μg/g.

INTRODUCTION

Prior to further development and testing in humans, extensive testing of a promising new drug in animals is necessary to determine toxicological properties and any short- and long-term adverse effects. Such a study requires an analytical method to assess the concentration, uniformity and stability of the drug when mixed with a carrier (feed) for administration to animals. Not only does the method have to be specific for low levels of drug with respect to coextracted feed excipients, but also the stability-indicating requirement necessitates specificity with respect to known and postulated degradation products. Because of this demanding specificity requirement, a chromatographic technique coupled with specific detection is often required.

In liquid chromatography, solid electrode electrochemical detectors operated in the amperometric mode (usually monitoring oxidations) provide some degree of selectivity because the number of electroactive compounds is limited and because the applied potential can be adjusted to make the detector respond to some electroactive compounds and not to others[1]. However, the latter becomes less useful when a high positive potential is required to detect the compound of interest, since all species with oxidation potentials below that applied will give a response.

The selectivity can be further enhanced in electrochemical detection by employing a differential pulse waveform in which the detector is responsive only to species which have oxidation potentials situated near to or between the initial and

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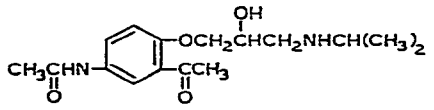
final potentials[2]. This potential window (modulation amplitude) generally ranges from 5 to 100 mV.

Differential pulse waveforms have been used with mercury-film[3] or mercury pool[4] detectors to enable species which have reduction potentials to be monitored. Recently, a convenient commercial dropping mercury electrode (DME) detector became available (*vide infra*) which adds the feature of a freshly renewed electrode surface to the extended negative potential range attainable.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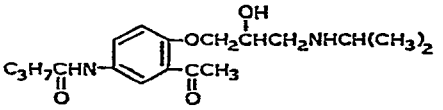
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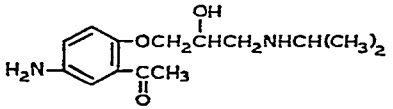
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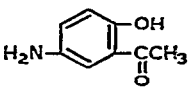
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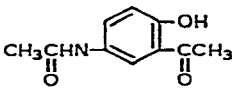
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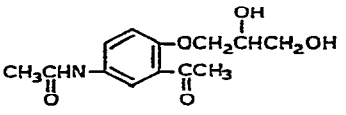

VI

This report describes an application of differential pulse polarographic liquid chromatographic detection with the DME for a stability-indicating assay of 0.005–1% diacetolol in feed. Diacetolol (I), the acetyl metabolite of the β-adrenergic receptor antagonist acebutolol (II)[5], is being studied as a possible anti-hypertensive agent. Thin-layer chromatographic[6,7], gas chromatographic[8] and reversed-phase high-performance liquid chromatographic (HPLC)[9,10] techniques have been developed for both acebutolol and diacetolol. However, none of the assays have been tested in feed analysis and have been proven specific for diacetolol with respect to a range of possible degradation products. The feed assay method reported here is specific for low levels of diacetolol with respect to coextracted feed excipients and known and probable degradation products. The latter include the acid hydrolysis product III[6] and related compounds: namely, the ether cleavage products IV and V, and the amine hydrolysis product VI.

EXPERIMENTAL

Instrumentation

A high-performance liquid chromatograph (ALC/GPC 202; Waters Assoc., Milford, MA, U.S.A.) equipped with a U6K injection valve, Model 6000 reciprocating pump, Model 440 absorbance detector at 254 nm (Waters Assoc.) and a slurry packed microparticulate reversed-phase column (Spherisorb ODS, 10 μm; Phase Separations,

Hauppage, NY, U.S.A.) was used. A flow-rate of 1.5 ml/min and in injection volume of 100.0 μl were used.

A Princeton Applied Research Model 310 DME polarographic detector controlled by a Model 174A polarographic analyzer served as the HPLC detection system. The analyzer was operated in the differential pulse mode with a modulation amplitude of 50 mV and an initial potential selected as described below. The detector was operated in the DME mode utilizing a small drop size and a drop time of 1 sec. The differential pulse polarographic work was done at a scan rate of 2 mV/sec. All potentials are reported *versus* a silver–silver chloride reference electrode saturated in KCl.

Preliminary work was also done with a glassy carbon thin-layer electrochemical detector (Model LC-16; Bioanalytical Systems, Inc., West Lafayette, IN, U.S.A.) operated in the amperometric mode.

Reagents, materials and eluent

Diacetolol (I), acebutolol (II), III, IV, V and VI were kindly supplied by May & Baker (Dagenham, Great Britain). Feed samples were prepared with Purina Formulab Chow. The mobile phase was methanol–aqueous 1% (w/v) ammonium acetate (35:65) which was filtered through a 0.5 μm Millipore filter and deaerated with nitrogen or helium prior to and during use.

Sample and standard preparation

About 5 g of feed sample were accurately weighed and transferred to a 2.0-cm I.D. glass column containing a small glass wool plug. Methanol was added and the stopcock adjusted to yield a flow-rate of 10–20 ml/min, then closed when the first 200.0 ml were collected. For 0.005% and 0.05% feeds, 10.0-ml and 1.0-ml aliquots, respectively, of the resulting eluent solution were evaporated to dryness at 50–60°C with a stream of nitrogen. For 1% feeds, the eluent solution was diluted 1.0 to 25.0 with methanol and a 1.0-ml aliquot evaporated to dryness. Exactly 3.0 ml of 0.01 *M* phosphoric acid were added to the residue, the resulting solution filtered with a Millipore filter and injected into the chromatograph.

A standard solution was prepared by accurately weighing about 80 mg of diacetolol reference standard, dissolving and diluting to 100.0 ml with water, and diluting 1.0 ml of this solution to 200.0 ml with 0.01 *M* phosphoric acid.

Sample analysis

The amount of diacetolol present in the feed sample, expressed as wt.%, was calculated from

$$\% \text{ diacetolol} = \frac{h_u}{h_{std}} \times \frac{W_{std}}{W_{spl}} \times F$$

where W_{std} is the weight in mg of the diacetolol standard used to prepare the standard stock solution, W_{spl} is the sample weight in g, h_u and h_{std} are the peak heights observed for the sample and standard solutions respectively and F is a dilution factor (F = 0.0003, 0.003 and 0.075 for 0.005%, 0.05% and 1% feeds, respectively).

Synthetic medicated feed samples

A 100-g quantity of 1% diacetolol in feed was prepared in the laboratory directly by weighing the appropriate quantities of pure drug and feed and mixing with a rotary mixer for 2 h. Similarly, 200-g quantities of 0.05% and 0.005% feed were prepared from the 1% and 0.05% feeds, respectively, by mixing the appropriate medicated feed with unmedicated feed. A portion of a large batch (65-kg) 1% feed sample used in an actual animal study was also obtained.

RESULTS AND DISCUSSION

A glassy carbon thin-layer electrochemical detector operated in the amperometric (oxidative) mode was initially tried. However, the high anodic potential required (1.5 V) resulted in excessive noise and significant excipient interference. Results obtained were similar to that obtained for the absorbance detector (see below). Operation of the glassy carbon detector in the highly selective differential pulse mode was prevented by the design of the cell (counter and reference electrodes located far downstream from the working electrode[11]), and operation in the reductive mode was precluded by the high baseline and excessive noise associated with the high cathodic potential required (−1.3 V).

Although the DME provided sufficient hydrogen overvoltage for monitoring the reduction of diacetolol, when operated in the current sampled d.c. mode at −1.4 V, samples containing less than *ca.* 1 μg injected could not be accurately determined (the nominal sample size at the lowest feed level required a 400-ng injection). However, the charging current discrimination properties of differential pulse detection at the DME lowered the detection limit to the 10 ng range.

Effect of pH on peak potential and peak current

Differential pulse polarograms were obtained from a solution of 40 μg/ml diacetolol in eluent with the pH adjusted to various values from 3.4 to 7.2 with glacial acetic acid. From a plot of peak current and peak potential *versus* pH (Fig. 1), a pH of 7.2 for the eluent (no acetic acid added) and a trial initial potential of −1.25 V for the polarographic HPLC detector were chosen. The initial potential was optimized by

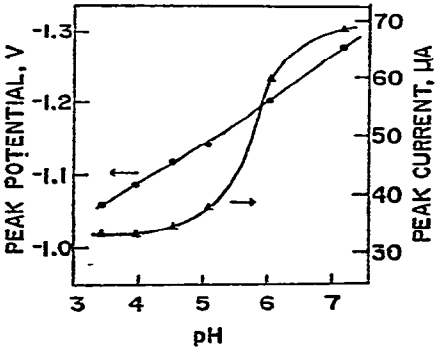


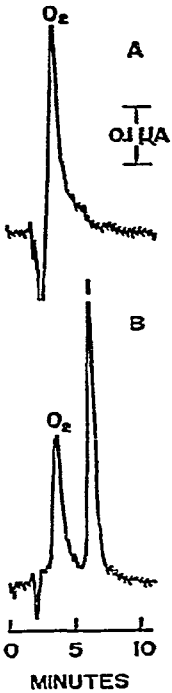
Fig. 1. Dependence of differential pulse peak current and peak potential on pH.

varying this parameter in increments of 25 mV while monitoring the diacetolol chromatographic peak height. The optimum initial potential (the initial potential which yielded the greatest peak height) slowly drifted to more negative values with time, *i.e.*, from −1.275 V to −1.375 V over a period of 4 months. This drift, which may have been caused by a slow change in liquid junction potential and/or salt concentration due to the transport of methanol across the reference electrode Vycor frit, required reoptimization of the initial potential every week or so. Replacing the reference electrode frit and filling solution returned the optimum initial potential to its original value.

Specificity

Chromatograms of a 0.005% feed sample and a corresponding unmedicated feed sample obtained with the polarographic detector operated in the differential pulse mode (Fig. 2) indicated no interference from feed excipients, although serious excipient interference was observed when the same solutions were examined with the absorbance detector at 254 nm (Fig. 3). (The signal at 254 nm was nearly 70% of that obtained at 233 nm, the absorbance maximum for diacetolol.)

The 0.04 Hz background noise observed with the polarographic detector (Fig. 2)



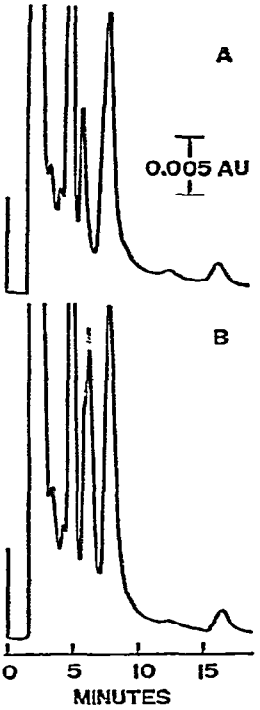


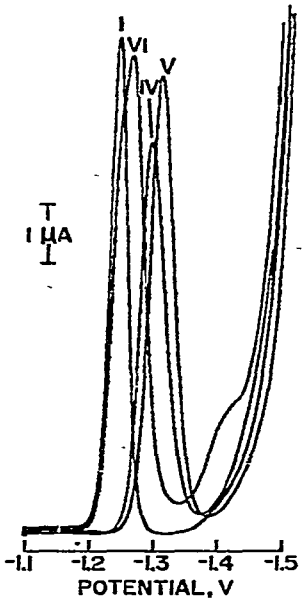
Fig. 2. Chromatograms of unmedicated feed (A) and 0.005% diacetolol (I) in feed (B) obtained with the polarographic detector. Both medicated and unmedicated feeds were carried through the same extraction procedure.

Fig. 3. Chromatograms of unmedicated feed (A) and 0.005% diacetolol (I) in feed (B) obtained with the absorbance detector at 254 nm.

was apparently HPLC pump noise. Since the noise posed no serious interference, no attempt was made to eliminate it. Each chromatogram also contained an oxygen peak (sample and standard solutions were not deaerated) which was well resolved from the diacetolol peak.

Differential pulse polarograms of 40 μg/ml solutions of the degradation products dissolved in eluent indicated that the polarographic detector, when optimized for diacetolol, should not be responsive to IV and V (Fig. 4). This was verified by chromatograms of a solution of 3 μg/ml diacetolol, 5 μg/ml acebutolol, 0.6 μg/ml III, 9 μg/ml IV, 9 μg/ml V and 12 μg/ml VI in 0.01 *M* phosphoric acid (Fig. 5). Compound V, which was detected by the absorbance detector (Fig. 5A), was not resolved from the diacetolol peak. However, when concentrations of V as high as 40 μg/ml (ten times the nominal diacetolol concentration) were injected, no response was obtained with the polarographic detector. Examination of the separation on another reversed-phase column (μBondapak C_{18}, Waters Assoc.) revealed that compound V was separated from diacetolol but compound IV (which was not detected polarographically) interfered. Compound VI, which according to Fig. 4 should yield a reduced polarographic detector response, was not resolved from the oxygen peak (Fig. 5). (Although III was not detected as a separate peak by the absorbance detector at 0.6 μg/ml, it was detected at higher concentrations.)

Specificity was further tested by attempted forced degradation of diacetolol.



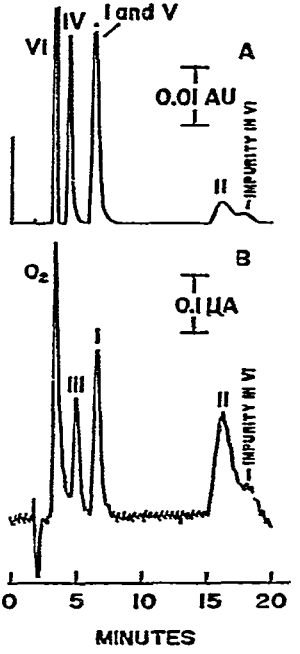


Fig. 4. Differential pulse polarograms of diacetolol (I) and possible degradation products. Polarograms of acebutolol (II) and compound III, which yielded peak potentials identical to that for diacetolol, are omitted for clarity.

Fig. 5. Chromatograms of diacetolol (I), acebutolol (II) and possible diacetolol degradation products obtained with the absorbance detector at 254 nm (A) and the polarographic detector (B).

A 0.005% feed sample stored in an oven at 65°C for 2 weeks yielded an assay value of about 50% of theory. No additional peaks were observed with either the polarographic or absorbance detector. A 0.05% feed sample stored in the oven at 120°C for 3 days did not yield a peak for diacetolol, although the pure drug stored under similar conditions yielded a single peak corresponding to 99.3% of theory. A chromatogram of a solution of diacetolol in 0.1 *M* HCl stored for 2 months at room temperature yielded an assay value of 47% of theory and an additional peak with a retention time identical to that for III. A chromatogram of a solution of the pure drug in 0.1 *M* HCl stored for 7 h at 37°C indicated that less than 1% had been converted to III. A chromatogram of a solution of diacetolol in 0.1 *M* NaOH stored for 6 days at room temperature yielded an assay value of 69% of theory and a peak corresponding to III. Examination of this solution with the absorbance detector yielded a small unknown peak prior to the diacetolol peak that was undetected by the polarographic detector. When the diacetolol peaks from the forced degradation study were separately collected, preconcentrated and spotted on fluorescent and non-fluorescent silica gel TLC plates according to the procedure of Steyn[6], the single spot observed in each case yielded good evidence that all of the decomposition products are separated by HPLC.

Method performance

A five-point plot of chromatographic peak current *versus* concentration in μg/ml was found to be linear from about 1 to 9 μg/ml with a correlation coefficient of 0.99995, a slope of 0.11 μA·ml/μg, a standard error of estimate ($S_{y/x}$) of 0.003 μA, a % intercept [(y-intercept/$\bar{y}$) × 100, where $\bar{y}$ is the average y] of −0.6% and a % variation [($S_{y/x}/\bar{y}$) × 100] of 0.6%. The polarographic detector linearity fell off at concentrations greater than 9 μg/ml although linearity of the absorbance detector was observed up to at least 85 μg/ml. A similar loss of linearity was observed for the oxidation of theophylline when a glassy carbon detector was operated in the differential pulse mode and was ascribed to the dependence of the half-wave potential on concentration caused by the complex and irreversible nature of the electrode reaction[11].

Six samples each of weights ranging from 80 to 120% of nominal sample size (5 g) from 0.005% and 0.05% synthetic feed preparations yielded the results in Table I.

TABLE I

ASSAY OF DIACETOLOL IN FEED SAMPLES

Sample Weight (g)	*0.005% Sample**		*0.05% Sample***		*1% Sample*	
	Found (%)	*Recovered (%)*	*Found (%)*	*Recovered (%)*	*Found (%)*	*Label (%)*
4.00	0.00504	99.6	0.0489	97.9	0.992	99.2
	0.00439	86.8	0.0493	98.6	1.034	103.4
5.00	0.00444	87.7	0.0493	98.6	1.037	103.7
	0.00536	105.9	0.0474	94.9	1.044	104.4
6.00	0.00441	87.2	0.0497	99.4	1.016	101.6
	0.00448	88.5	0.0482	96.5	1.027	102.7
Average	0.0047	92.6	0.0488	97.7	1.024	102.4
Relative S.D.	8.8%		1.7%		1.7%	

* 0.00506% theory.

** 0.0500% theory.

The 1% feed samples were obtained from a large batch mixed for an actual animal study.

In conclusion, the method is sufficiently precise for use without an internal standard and sufficiently sensitive to determine diacetolol in feeds at levels as low as 0.005% with reasonable accuracy. The use of a polarographic detector in the differential pulse mode holds considerable promise for feed assays which require a high degree of specificity with respect to degradation products and excipients.

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